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G2/M checkpoint associated repression of polo-like kinase-1 mediated by the tumour suppressor, p53

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Lynsey McKenzie

2010

University of Dundee

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**G2/M Checkpoint Associated
Repression of
Polo-like Kinase-1 Mediated by the
Tumour Suppressor, p53**

**A thesis submitted for Doctor of Philosophy
at The University of Dundee**

July 2010

Lynsey McKenzie

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Abbreviations

aa	amino acid
Amp	Ampicillin
Apaf1	Apoptosis protease activating factor 1
APC	Anaphase Promoting Complex
APS	Ammonium Per sulphate
ARF	Alternate Reading Frame (of the CDKN2A locus)
ATP	Adenosine Tri-Phosphate
ATR	Ataxia-Telangiectasia and Rad3 Related
ATRIP	ATR Interacting Protein
ATM	Ataxia-Telangiectasia Mutated
BAD	Bcl-2 Associated Death Receptor homolog
BAX	Bcl-2 Associated X Protein
Bcl	B Cell Lymphoma
BER	Base Excision Repair
BID	BH3 Interacting Domain Death agonist
bp	base pair
BRCA	Breast Cancer type 1 susceptibility protein
BSA	Bovine Serum Albumin
°C	Degree Celsius
c-Abl	cellular counterpart to the virally encoded gene, v-Abl (Abelson murine leukaemia viral oncogene)
C-terminus	Carboxyl Terminus
CBF	CCAAT Binding Factor
CBP	cAMP Response Element Binding (CREB) Binding Protein
cdc	cell division cycle
CDC25C	Cell Division Cycle 25 homolog C
CDE	Cell cycle-Dependent Element
CDK	Cyclin Dependent Kinase
CHK1	Checkpoint Kinase 1
CHK2	Checkpoint Kinase 2
ChIP	Chromatin Immunoprecipitation
CHR	Cell cycle genes Homology Region

CIP1	CDK-Interacting Protein 1
CK	Casein Kinase
Cop1	Constitutive Photomorphogenesis protein 1
CP1	CCAAT binding Protein
CPD	Cyclobutane Pyrimidine Dimers
CSD	Cytoplasmic Sequestration Domain
Da	Dalton
DBD	DNA Binding Domain
DDB2	DNA Damage Binding Protein 2
ddH ₂ O	Double distilled water, milli-Q water
DISC	Death Induced Signalling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DNA-PK	DNA-Protein Kinase
DSBs	Double Strand Breaks
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EMI1	Early Mitotic Inhibitor 1
EMSA	Electro-phoretic Mobility Shift Assay
FADD	FAS-associating Death Domain protein
FAT	Transcriptional factor Acetyl-Transferase
FBS	Fetal Bovine Serum
FLICE	FADD-like ICE
GADD45	Growth Arrest and DNA Damage inducible factor
GGR	Global Genome Repair
GSK3	Glycogen Synthase Kinase
h	hour
HAT	Histone Acetylase
HDAC	Histone Deacetylase
HR	Homologous Recombination
IAP	Inhibitors of Apoptosis
IR	Ionising Radiation
kDa	Kilo-Dalton
l	Litre
LB	Luria-Bertani medium
Luminol	3-Aminophthalhydrazide
m	Milli

M	Molar
μ	Micro
MAD1	Mitotic Arrest Deficient protein 1
MDM2	Murine Double Minute 2 protein
min	Minute
MMR	Mismatch Repair
MMTV	Mammary Tumour Virus
mol	Mole
MPT	Mitochondrial Permeability Transition
MRN	Mre11, Rad50 and Nbs1 complex
Myt7	Membrane-associated Tyrosine- and threonine-specific cdc2-inhibitory kinase
N-terminus	Amino-terminus
NER	Nucleotide Excision Repair
NES	Nuclear Export Signal
NF-Y	Nuclear Transcription Factor Y
NHEJ	Non-Homologous End Joining
Nlp	Ninein-like protein
NLS	Nuclear Localisation Signal
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP) Ribose Polymerase
PBD	Polo-Box Domain
PBS	Phosphate Buffered Solution
PCAF	P300/CBP-Associated Factor
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
RFC	Replication Factor C
PIKK	Phosphatidylinositol-3-Kinase-like Kinase
Pirh-2	p53-induced protein with RING-H2 variant
Pol	Polymerase
PRC1	Protein Regulator of Cytokinesis 1
PTEN	Phosphatase and Tensin homolog
PTGF	Platelet-derived growth factor
Pu	Purine
PUMA	p53 Up-regulated Modulator of Apoptosis
Py	Pyrimidine
Rb	Retinoblastoma associated protein
RING	Really Interesting New Gene
RPA	Replication Protein A
RT	room temperature

rpm	revolutions per minute
SDS	Sodium Dodecyl Sulfate
sec	second
Smad	Mothers against DPP (Mad) and Sma human homolog
SOB	Super Optimal Broth
SOC	Super Optimal Culture
SP1	Specificity Protein 1
ssDNA	single stranded DNA
SV40	Simian Virus 40
SWI/SNF	SWItch/Sucrose NonFermentable
TAD	Trans-activation Domain
TAF	Transcription Activation Factor
TBP	TATA Binding Protein
TCR	Transcription Coupled Repair
TD	Tetramerization Domain
TFIID	Transcription Factor II D
TGF	Transforming Growth Factor
TLS	Translesion Synthesis
TNF	Tumour Necrosis Factor
TRADD	Tumor Necrosis factor Receptor type 1-Associated Death Domain protein
Tris	Tris (Hydroxymethyl) Aminomethane
Tween 20	Polyoxyethylenesorbitan Monolaurate
UBC	Ubiquitin conjugating enzyme
UV	Ultra Violet
V	Volts
v/v	volume to volume
w/v	weight to volume
XPC	Xeroderma Pigmentosum group C

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I would like to thank my parents and my family for the huge and constant moral support they have given me. Lastly and most importantly, I thank my fiancé Vincent Rao for his incredible patience, continual encouragement and unwavering faith and confidence in me.

Declaration

I declare that the following thesis is based on the results of investigations conducted by myself, and that this thesis is of my own composition. Work other than my own is clearly indicated in the text by reference to the relevant researchers or to their publications. This dissertation has not in whole, or in part, been previously submitted for a higher degree.

Lynsey McKenzie

July 2010

The work presented in this thesis has been carried out by Lynsey McKenzie under my supervision. I confirm that she has fulfilled the conditions of the relevant ordinance and regulations at the University of Dundee for the degree of Doctor of Philosophy.

Doctor David W. Meek

July 2010

Abstract

Polo-like Kinase-1 (PLK1) is an important mediator of the G2/M phase of the cell cycle that is down-regulated in a DNA damage dependent manner. In cancer cells PLK1 is overexpressed and allows for continued proliferation of the cell by overriding this checkpoint. Here I show that PLK1 is down-regulated in a p53-dependent manner and that can occur both in response to DNA damage and to a non-genotoxic stimulus of the p53 pathway. My data show that p53 is able to repress PLK1 through a responsive element in the promoter and that p53 is necessary and sufficient to cause PLK1 repression. When examined in the context of a PLK1 promoter/reporter fusion, wild type but not mutated forms of p53 can repress expression. EMSA shows that p53 binds to the p53-responsive element and that mutation of this element reduces p53 binding. Furthermore, PLK1 repression occurs independently of p21-mediated arrest at G1/S, a stage of the cell cycle where PLK1 levels are physiologically low. PLK1 repression mediated by p21 through the CDE/CHR element in the promoter does not appear to cause significant repression of PLK1 but may play a minor role. Down-regulation of PLK1 is relieved by the HDAC inhibitor TSA and supports the transcriptional repression mechanism described in this thesis. Silencing of PLK1 expression by siRNA interferes with cell cycle progression consistent with a role in the p53-mediated checkpoint. This thesis provides two distinct and perhaps overlapping mechanisms by which p53 may repress PLK1: 1) through competitive displacement of an unidentified transcription factor that is essential for normal PLK1 expression and 2) through HDAC recruitment leading to local repression-associated changes in the chromatin structure. These data establish PLK1 as a transcriptional target of p53 that is required for efficient G2/M arrest.

Chapter 1: Introduction

1.1 p53, Guardian of the Genome

“If genius is the ability to reduce the complicated to the simple, then the study of p53 makes fools of us all” (Vousden and Prives, 2009).

1.2 Discovery of p53

The study of p53 began thirty years ago with the identification of a protein that was found bound to the SV40 Large T-antigen and which ran on an SDS-PAGE gel at a molecular weight of around 53 kDa (Lane and Crawford, 1979, Linzer and Levine, 1979). Furthermore, this transformation related protein was found at high levels in tumours whereas it was not observed in normal tissue (Rotter, 1983). Other authors also showed that there were low levels of p53 in non-transformed cells and high levels in transformed cells (DeLeo *et al.*, 1979) and interestingly p53 was found in the nucleus in transformed fibroblasts and in the cytoplasm of non-transformed fibroblasts (Rotter *et al.*, 1983). On the basis of the above information and the fact that p53 was found bound to this viral protein, which is a known oncogene product, at high levels in SV40 transformed cells and also in other non-transformed types of cancer cells, p53 was considered an oncogene itself (for review see (Levine and Oren, 2009)).

Although during this time data shown by other authors suggested p53 was not an oncogene, for example data that showed that *Trp53* (*Transformation related protein 53*) produced aberrant mRNA due to retroviral insertion of DNA into the first intron of the gene in an Abelson murine leukaemia virus transformed mouse cell line (Wolf and Rotter, 1984) p53 was continued to be considered an oncogene until the

realisation that earlier experiments used cDNA clones that encoded mutant p53. In fact, wild type p53 repressed the transformation of cultured cells (Finlay *et al.*, 1989) and mutation of p53 is required for transformation of cells, alongside the *ras* oncogene (Hinds *et al.*, 1989). From these and other experiments carried out by different groups p53 was re-defined as an anti-oncogene (or recessive oncogene).

1.3 Tumour Suppressor

Half of all human cancers have a mutation in p53 (Greenblatt *et al.*, 1994). The other 50% of human cancers are found to have mutations in proteins regulating p53, for example MDM2 (as discussed later in this Chapter). Current success in restoring p53 function in tumour cells, by the introduction of small molecules that stabilise p53, makes p53 an attractive therapeutic target in the fight against cancer (Brown *et al.*, 2009). There are currently over 50,000 papers in the study of p53. To understand the impact of the absence of p53 in tumour progression, p53 tumour suppression has been studied in many mouse models. As reviewed in Attardi *et al.*, 1999, the first p53 null mouse was described as developmentally normal but with a higher susceptibility to tumours and at an early age (Donehower *et al.*, 1992). p53 heterozygote mice were also described, and have been represented as the mouse model for patients with Li-Fraumeni syndrome, a syndrome in which humans have one allele of wild type p53 and one of mutant p53 and are highly susceptible to tumour formation (Malkin *et al.*, 1990, Varley *et al.*, 1997a, Varley *et al.*, 1997b). Mouse studies, involving the crossing of p53-null mice and other cancer prone mice, have also revealed the importance of other tumour suppressors that interact with p53 in tumour suppression and also revealed oncogenes involved in tumour development (for review see

(Attardi and Jacks, 1999). For example, Rb (Retinoblastoma) heterozygote mice crossed with p53 null mice, produced progeny that showed accelerated tumourigenesis compared to that of the Rb-null alone or p53 wild type alone progeny, suggesting that Rb and p53 cooperate in tumour suppression. An example of mice models that have revealed oncogenes is described, for example, by Hundley *et al.*, 1997. These authors describe that the crossing of MMTV *ras* transgenic mice and p53 null mice, give progeny that have an increased instance of tumour progression which was faster and earlier in onset than the p53 wild type mice. Their data further showed that *ras* is an oncogene, and when in p53 null mice, leads to aggressive tumour formation. In conclusion p53 acts as a barrier to tumourigenesis.

Overall these mouse studies were vital to defining p53 as a tumour suppressor and the role p53 plays in tumour suppression. Indeed, Aranda-anzaldo and Dent, 2007, have argued that p53 has been selected mainly for its role in development. They argue that since 1) short-lived organisms don't need the tumour suppressor roles of p53 and 2) p53 tumour suppression is only needed species that live for a long time post reproductive age, therefore the development role of p53 must have been selected for long before any tumour suppressor role of p53. However this would appear counterintuitive since natural selection would not select for traits that occur post reproductive phase. Furthermore the tumour suppressive role during development is needed during development as p53 mutations are found in childhood cancers (Chompret *et al.*, 2000) opposing the theory that p53 mutation only occurs after the age of reproduction. Therefore p53 has a role to play in tumour suppression in individuals that have not surpassed the age of reproduction and this suggests that tumour suppression is the function of this protein.

1.4 The Transcription Factor

The p53 tumour suppressor is a transcription factor that binds to sequence specific DNA at the promoters of genes and also at the non-coding regions of gene (introns) (Kern *et al.*, 1991). p53 activates transcription via its trans-activation domains (Chang *et al.*, 1995, Fields and Jang, 1990, Raycroft *et al.*, 1990, Walker and Levine, 1996). The structure of p53 is divided in to three functional domains: the aforementioned trans-activation domains are situated at the N-terminus; a DNA binding domain; and a C-terminal domain containing a tetramerization domain, nuclear localisation signals (NLS'), nuclear export sequence (NES') and regulatory domain, as shown in Figure 1.1.

The p53 Protein Domains

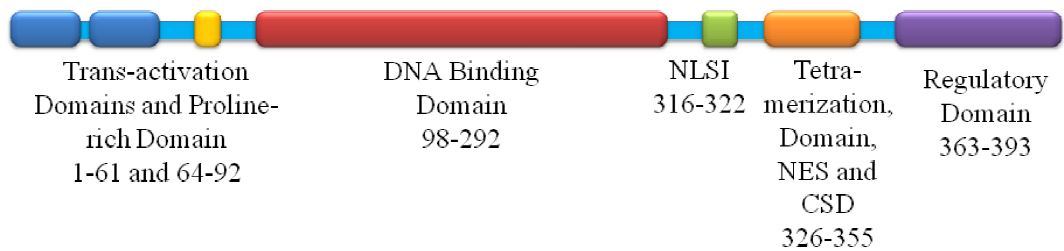


Figure 1.1: The protein domains of p53

The p53 protein consists of three main domains: the trans-activation domain, DNA binding domain and C-terminal domain. The C-terminal domain consists of NLS', NES, tetramerization domain and regulatory domain.

The p53 protein consists of 393 aa residues and forms a tetramer. The crystal structure of the wild type p53 and mutant bound DNA complexes were refined by

the Fersht laboratory, who show that each monomer binds to a 5 bp sequence (Tidow *et al.*, 2007). These authors show that the p53 tetramer forms a tunnel, with the DNA binding domain (or core domain, residues 100-300) at one end and the tetramerization domain in the C-terminus at the other. The binding of p53 to DNA is shown in Figure 1.2 and linking the two domains are four unstructured chains (Shakked, 2007).

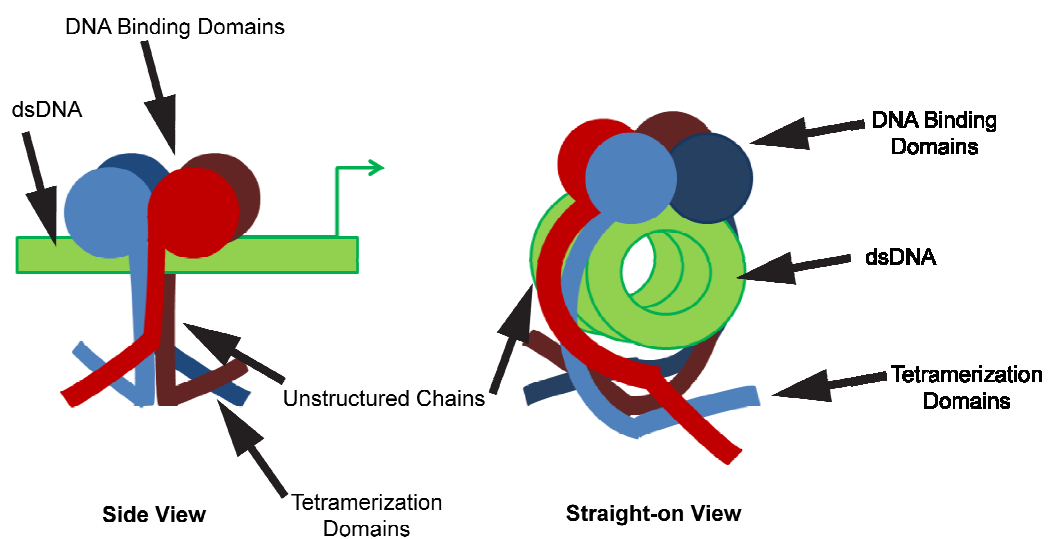


Figure 1.2: p53 forms a “tunnel” structure around DNA

The structure of p53 bound to DNA shows that p53 forms a tunnel formation around DNA. The side view shows the DNA binding domains on top of the DNA which are linked to the tetramerization domains at the bottom via unstructured chains. The straight-on view shows this structure from the front and shows the tunnel formation around the DNA. This Figure has been modified from Shakked, Z., 2007. Notably the N-terminus (not shown) does not participate in the binding of p53 to DNA.

The next three Sections (Sections 1.4.1-1.4.3) describe in more detail the main roles of these domains.

1.4.1 Trans-activation Domain

Activation of transcription by p53 in part occurs through the interaction of the trans-activation domain (TAD, residues 1-42) in the N-terminus of p53 with the TATA-Binding Protein (TBP) (Martin *et al.*, 1993) and consequently the general transcription factor TFIID complex, which consists of TBP and other Transcription Activation Factors (TAFs), (Farmer *et al.*, 1996, Liu *et al.*, 1993) such as TAF1 and TAF(II)250. This more stable complex stimulates the binding of p53 to a sequence specific DNA binding site (Chen *et al.*, 1993). Interestingly, inactivation of TAF(II)250 affects the p53-MDM2 auto-regulatory loop (described in the next Section) causing a decrease in the levels of MDM2 and activation of the p53 pathway (Wasylyk and Wasylyk, 2000). p53 stimulates the TFIID-TFIIA-promoter complex assembly by inducing a conformational change in the complex (Xing *et al.*, 2001). Xing *et al.*, 2001, also show the mechanism by which the SV40 Large T-antigen induces transformation through inhibition of p53. They show that the Large T-antigen, which once bound to the DNA via p53, represses transcription by preventing TBP from binding to the TATA box and ultimately inhibits TFIID-TFIIA complex assembly, needed for transcription.

Co-activators of p53 are also required for p53 activated transcription, such as the CREB binding protein (CBP) and p300 (both of which are transcriptional co-activators and histone acetyl-transferases). The p300/CBP complex directly interacts with the p53 TAD (TAD1 sub-domain) and stimulates p53 mediated expression of genes such as *MDM2* (Gu *et al.*, 1997). This was the first description that p53 activates expression of its negative regulator. Mutation of residues 22 and 23 in TAD1 of human p53 abolishes the interaction of p300/CBP to p53 and abolishes p53

induced transcription (Gu *et al.*, 1997). Notably, residues 22 and 23 are the initial two residues of an LXXLL motif (which forms an α -helix in the DNA); a motif necessary for the binding of p300 to p53. Phosphorylation of residues in p53, including T18 and S20, are also important in p300 binding and activate p53 by stabilising its binding to p300 (Dornan and Hupp, 2001). In addition to this motif, the proline repeat motif (proline repeat domain) or proline-rich domain (as shown in Figure 1.1) is required for p300 acetylation of p53 (Dornan *et al.*, 2003). Dornan *et al.*, 2003 suggest a mechanism whereby the binding of p53 to DNA induces a conformational change in p53, which opens up its C-terminus to p300 mediated acetylation. Deletion of the LXXLL motif inhibits the binding of p300 to DNA bound p53 and p300 acetylation of p53. Other studies further validate the interaction of p53 with p300/CBP and show that the adenovirus viral oncogene E1A inhibits this interaction, which may be part of the mechanism by which E1A induces cellular transformation (Lill *et al.*, 1997).

1.4.2 DNA Binding Domain

As p53 is a transcription factor, its primary function is to activate and repress gene expression. p53 binds to DNA at sequence specific elements, termed p53 responsive elements, through its site-specific DNA binding domain (DBD, residues 98-292). The p53 responsive element generally consists of the two half site sequences (each 10 bp), separated by a spacer of usually 0-13 bp: half site sequence - 5'PuPuPuC(A/T)(T/A)GPyPyPy-3' (Pu = Purine (A/G), Py = Pyrimidine (T/C)) (el-Deiry *et al.*, 1992) (for review see (Riley *et al.*, 2008)). The half site provides the binding site for one p53 dimer, which mediates the binding of the second p53 dimer

to the second half site to take place, with the outcome of bending of the DNA. Therefore at the DNA level p53 is able to form into a tetramer (Kitayner *et al.*, 2006), where each p53 monomer binds to 5 bp of a half site sequence, or quarter site.

p53 responsive elements (or binding sites) are complex and varied. The orientation of quarter sites need not be symmetrical, for example the p53 binding sites in the p21 promoter, which include a p53 binding site which is made up of two generic half sites, as described above and another which consists of two half sites, with a variability in their quarter sites: AGPyPuPu PuPuPuCA and TGPYPyPy PuPuPuCA (el-Deiry *et al.*, 1995). However, more recent data show that there are many more p21 promoter p53 binding sites and the orientation of their particular quarter sites are also very complex (Saramaki *et al.*, 2006). Interestingly p53 binding sites can be found up to 4 Kb upstream of the transcriptional start site of the p21 gene. p21 promoter p53 binding sites are only some examples of the kind of variability that can occur in the p53 binding site and there are many more binding sites in hundreds of other gene promoters. These are summarized comprehensively in Riley *et al.*, 2008.

There is much complexity that surrounds the consistency of a p53 responsive element. Another factor to be taken into consideration is the variability in length of the spacer between half sites. Introduction of one or more nucleotides into the spacer reduces p53 trans-activated gene expression, for example the introduction of an extra nucleotide into a spacer between two p53 responsive elements in the promoter of p21 reduces p53 mediated p21 expression significantly (Jordan *et al.*, 2008).

1.4.3 C-terminal Domain

The C-terminus of p53 consists of smaller domains and elements, including the tetramerization domain (TD, residues 326-355), nuclear localisation signals (NLS), nuclear export signal (NES) and regulatory domain (residues 363-393). The tetramerization domain is the site of oligomerization of p53 subunits and permits tetramer DNA binding, protein-protein interactions and p53 degradation (for review see (Chene, 2001)). Studies have shown that even though p53 can bind to DNA in the absence of the TD, its affinity for DNA is 10 to 100 times lower than the full length p53 (Balagurumoorthy *et al.*, 1995). Furthermore, the DNA bending that is caused by the binding of tetrameric p53 to DNA is unlike that of the binding of one monomer of full length p53 (Nagaich *et al.*, 1999, Pan and Nussinov, 2007). These data suggest that the TD stimulates both the strength of binding and the change in conformation of the DNA, needed for transcription by p53.

Other domains found in the C-terminus include the NES, which is important for the degradation of p53. In unstressed cell, the p53 regulatory protein, E3 ligase MDM2, binds to and ubiquitylates p53 in the DBD and C-terminus. The mechanism behind this is that MDM2 binds to p53 which causes a conformational change in p53 that exposes certain lysines for ubiquitylation (Sasaki *et al.*, 2007). Ubiquitylation then exposes the NES for nuclear export of p53 (Nie *et al.*, 2007). Further regulation of p53 through the NES, (found at the C-terminus of p53, residues 340-351), suggested by Stommel *et al.*, 1999, occurs through the tetramerization domain of p53. These authors show that the p53 NES alone is sufficient to mediate export of p53 from the nucleus and mutation of residues in the NES reduces p53 tetramerization (Stommel *et al.*, 1999). Furthermore, p53 export is inhibited by the addition of peptides that are

of the same sequence to that of the NES and tetramerization domains of p53. Therefore these data suggest a model where p53 tetramerization occludes the NES and attenuates nuclear export.

Another domain in the C-terminus that is important for the localisation of p53 is the NLS. Shaulsky *et al.*, 1990 show that there are three NLS sequences in the C-terminus of p53 and NLSI, found at the N-terminus end of the C-terminus, is the predominant signal. All three actively mediate nuclear localisation of p53 and NLS deprived wild type p53 could not migrate into the nucleus (Shaulsky *et al.*, 1990). In addition to the NLSs, other authors report the dependency of two *cis acting* domains, in the C-terminus, that also regulate p53 cellular localisation: a lysine-arginine element (residues 305-306) and a cytoplasmic sequestration domain (CSD, residues 326-355) (Liang and Clarke, 1999). These authors demonstrate that NLSI (the more prominent and efficient of the NLS' in p53) and lys-arg element form a bipartite NLS that is regulated by the CSD, which controls the binding of the NLS to the Importin α shuttling protein.

There are many domains and elements in the C-terminus, as discussed above, and in addition to these is the regulatory domain. This domain is regulated by various post-translational modifications including acetylation by p300/CBP proteins and ubiquitylation by MDM2 (Gu and Roeder, 1997, Lavin and Gueven, 2006). The C-terminus of p53 participates in the ability of p53 to be degraded by MDM2 as data show that the deletion of this region stabilized p53 without abrogating its ability to bind to MDM2 (Kubbutat *et al.*, 1998). Furthermore, the mutation of 6 lysines simultaneously (residues 370, 372, 373, 381, 382, and 386) increases the transcriptional ability of p53, suggesting that mutation of these residues increases

p53 stability by inhibiting degradation mediated by MDM2 (Rodriguez *et al.*, 2000). Interestingly, five of these residues (residues 370, 372, 373, 381 and 382) are targets of p300 mediated acetylation, which increases p53 sequence specific DNA binding activity (Gu and Roeder, 1997). Therefore it was assumed that acetylation regulates ubiquitylation and indeed this was shown to be the case. Li *et al.*, 2002, show that acetylation of p53 inhibits MDM2 ubiquitylation of p53. Furthermore they show that even under unstressed conditions acetylation can inhibit ubiquitylation (Li *et al.*, 2002). These data together show the complex interplay between p53 post-translational modifications that regulate p53 stabilisation and degradation.

The three main protein domains of p53 are targets of many proteins and post-translational modifications, some of which are mentioned above, and which regulate p53. A key example is the regulation of p53 by the oncogene, MDM2, and this is discussed in Section 1.5.

1.5 Regulation of p53

In unstressed conditions p53 is found at very low levels and undergoes rapid turnover. When cells are subjected to stress, levels of p53 increase and activate a cellular response, such as senescence or apoptosis, depending on the type and strength of stress and how much DNA damage has occurred. The principal mechanisms that control the p53 induction process are as follows:

1.5.1 Interaction of p53 and MDM2

Under normal circumstances, p53 is regulated by many proteins including the E3 ubiquitin ligase, MDM2 (See Figure 1.3) (notably other E3 ligases include Cop-1 and Pirh-2 (Harris and Levine, 2005)). MDM2 binds to p53 and mediates the degradation of p53 by the proteasome. The model of MDM2 regulation involves the binding of the p53 binding domain of MDM2 to the trans-activation domain of p53, in doing so blocking the interaction of p53 with proteins and factors required for p53 mediated transcriptional activation (Momand *et al.*, 1992). Once bound to this domain, a second binding between the acidic domain of MDM2 to the DNA binding domain of p53 occurs (Yu *et al.*, 2006), which is essential for the ability of MDM2 to ubiquitylate p53 (Kawai *et al.*, 2003, Meulmeester *et al.*, 2003, Wallace *et al.*, 2006). Interestingly, the phosphorylation status of the acidic domain is very important for MDM2 mediated ubiquitylation, as data show that the mutation of certain serine residues in this domain reduces or abolishes the degradation of p53 by MDM2, even though the mutants retained ubiquitylation capacity (Blattner *et al.*, 2002). Many protein kinases are responsible for the phosphorylation of the acidic domain, such as CK2 (Casein Kinase 2), which can be recruited by the transcription factor, TAF(II)250 (Allende-Vega *et al.*, 2008). Others kinases that regulate MDM2 and p53 include GSK3 and CK1. GSK3 inhibition inhibits MDM2 dependent p53 degradation which interestingly does not inhibit the binding of the MDM2 to p53 (Kulikov *et al.*, 2005) and indicates that GSK3 negatively regulates p53 by inducing MDM2 binding. Similarly, CK1 RNA knock-down activated p53 (Huart *et al.*, 2009). It is shown that under stressed conditions CK1 phosphorylates MDM2 (Winter *et al.*, 2004) and is found bound to MDM2 promoting its binding to p53 (Huart *et al.*, 2009). However, under stressed conditions, CK1 phosphorylates p53

(MacLaine *et al.*, 2008, Sakaguchi *et al.*, 2000) and in doing so abrogates the binding of p53 to MDM2. Thus CK1 regulates both MDM2 and p53, depending upon whether the cell is under stress.

The third most important and required domain of MDM2 in the regulation of p53 is the RING finger (Fang *et al.*, 2000, Honda *et al.*, 1997). Studies suggest that, as an E3 ligase, MDM2 is responsible for the mono-ubiquitylation of p53 (see Figure 1.3) (Lai *et al.*, 2001) at six individual lysine residues in the C-terminus (Rodriguez *et al.*, 2000). However, other studies show that low levels of MDM2 induce mono-ubiquitylation and nuclear export of p53, whereas high levels promote the poly-ubiquitylation and nuclear degradation of p53 (Li *et al.*, 2003). Li *et al.*, 2003, reported that a p53-ubiquitin fusion protein that mimics mono-ubiquitylated p53 accumulated in the cytoplasm in an MDM2 independent manner and therefore their model suggests that mono-ubiquitylation is required for nuclear export of p53 only.

Generally, ubiquitylation of proteins is thought to require an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2 or UBC), and an ubiquitin ligase (E3). E1 binds and activates ubiquitin through the action of ATP. Ubiquitin is then transferred from an E2 in an E3 dependent manner, to a lysine residue on the target substrate (for review see (Pickart, 2004). Intriguingly, the type of poly-ubiquitylation decides the targets outcome, for example, K48-linked poly-ubiquitin chains target a substrate for degradation by the proteosome (Finley *et al.*, 1994) (for review see (Pickart, 1997)). Studies show that K63 is used as a linkage site in the formation of poly-ubiquitin chain structures that play an important role in DNA repair (Spence *et al.*, 1995).

Other ligases, such as the intrinsic ubiquitin ligase activity of p300, are also required for subsequent poly-ubiquitylation and ubiquitylation mediated proteasomal degradation (Grossman *et al.*, 2003). As illustrated by these authors, in vitro, p300 and MDM2 together, induced p53 poly-ubiquitylation, whereas MDM2 alone could only induce p53 mono-ubiquitylation. This data alongside data that show MDM2 has a p300 binding site in its acidic domain (Grossman *et al.*, 1998), put forward a model that p300 interacts, specifically and independently, with both p53 and MDM2 and regulates MDM2 mediated p53 degradation and turnover.

Evidence of the action of MDM2 as a negative regulator of p53 comes from the fact that MDM2 knockout mice have an embryonic lethal phenotype, however this lethality can be rescued by knockout of p53 (Jones *et al.*, 1995, Montes de Oca Luna *et al.*, 1995). Consistent with the model that MDM2 negatively regulates p53 is that the mechanism of induction of p53 requires that the MDM2-p53 interaction must be disrupted.

Further regulation of p53 comes in the form of an auto-regulatory loop, as shown in Figure 1.3. When p53 is not bound to MDM2, p53 is able to perform its function as a transcription factor and activate or repress down-stream target genes. Interestingly, one of these genes is *MDM2*. By activating the transcription of *MDM2*, MDM2 levels increase and in turn MDM2 binds to p53. The binding of p53 to MDM2 mediates the proteasomal degradation of p53, and itself (Fang *et al.*, 2000), and levels of p53 decrease. In turn p53 can no longer activate expression of *MDM2* and *MDM2* levels decrease (Wu *et al.*, 1993). This auto-regulatory feedback loop is the principal means of regulating the levels of both p53 and MDM2.

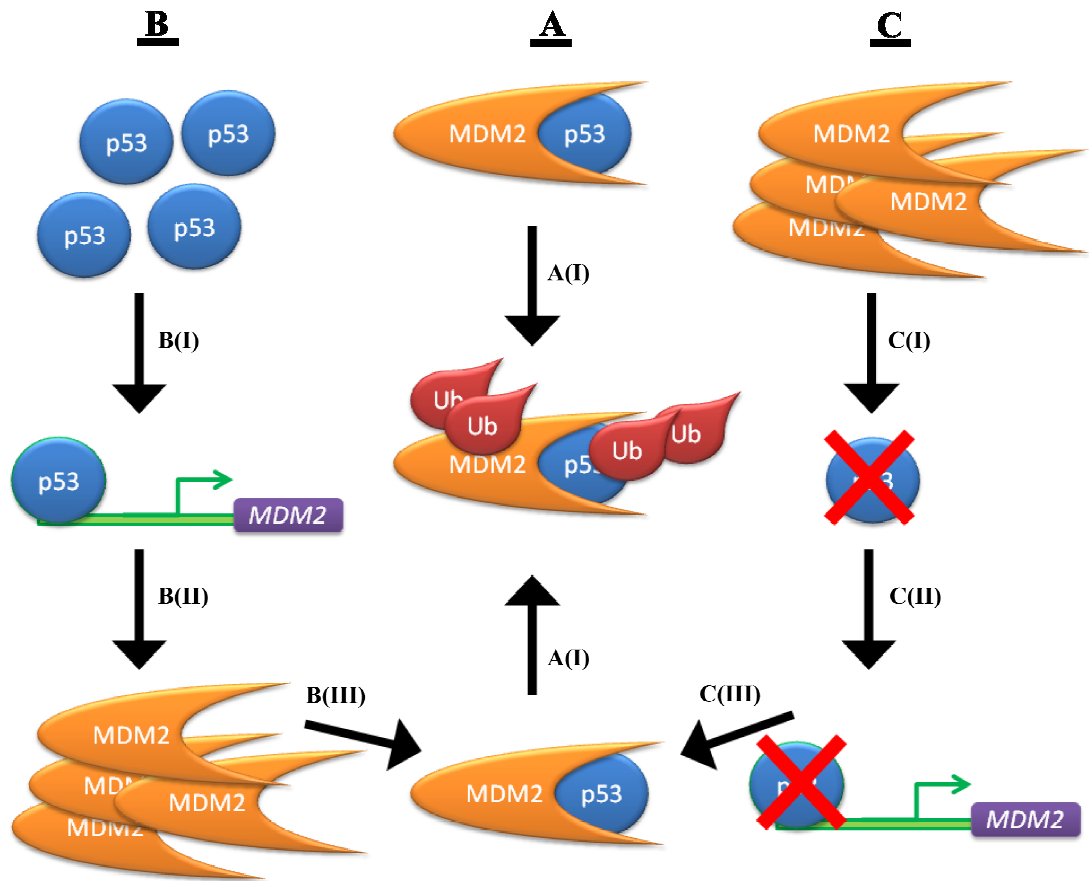


Figure 1.3: The p53-MDM2 auto-regulatory feedback loop

The p53-MDM2 auto-regulatory feedback loop allows p53 to be regulated in unstressed cells. As shown, MDM2 is bound to p53 via various interactions (pathway A) and ubiquitylates p53, and itself, mediating proteasomal degradation of both MDM2 and p53 (A(I)). Should levels of p53 increase (pathway B), the transcriptional function of p53 activates expression of MDM2 (B(I)) and therefore levels of MDM2 increase (B(II)). The higher levels of MDM2 are able to bind to the increased levels of p53, therefore lowering the level of p53 (B(III)). On the other hand, should levels of MDM2 increase, MDM2 inhibits p53 by binding to a greater proportion of p53 molecules (C(I)) and inhibits transcriptional activity of p53 and expression of MDM2 (C(II)). MDM2 levels decay over time and levels of MDM2 gradually decrease (C(III)).

In summary, there is very tight regulation of p53 by MDM2 under unstressed conditions, particularly in post-stress conditions. Various studies show that MDM2 is a potentially oncogenic protein, for example, the overexpression of MDM2 resulted in the immortalization of primary rat embryo fibroblasts and, alongside the activated *ras* gene, in the transformation of these cells (Finlay, 1993). In addition to this, introduction of the *MDM2* gene into non-transformed cells induced tumour

formation further suggesting that MDM2 is in fact an oncogene (Fakharzadeh *et al.*, 1991). Consistent with these findings, levels of MDM2 are increased in a number of cancers (Abdel-Fattah *et al.*, 2000, Reifemberger *et al.*, 1993)

In order for the p53 levels to increase and therefore determine cell fate after DNA damage, MDM2 regulation must be disrupted.

1.5.2 Stabilisation of p53 through the disruption of the p53-MDM2 complex

Under stressed conditions (see Section 1.5), the p53 pathway (see next Section) is activated and allows stabilisation of p53 and increase in levels of p53. But, how can this occur if MDM2 tightly regulates p53, keeping levels low and the protein inactive? The answer to this lies in the auto-regulatory loop. DNA damage activates many proteins which cause post-translational modification changes of p53 and MDM2 causing inhibition of the ubiquitylation of p53 by MDM2, the inhibition of the binding of the p53 TAD to the p53 binding site of MDM2 and inhibition of the interaction between the MDM2 acidic domain to the DNA binding domain of p53.

Phosphorylation is the first key step in the stabilisation of p53 (see Figure 1.4). As already mentioned, several phosphorylation sites in the p53 trans-activation domain of p53, including S15, T18 and S20 are phosphorylated in response to stress. The phosphorylation of S15 induces a conformational change in the p53 protein and reduces the binding of MDM2 (Shieh *et al.*, 1997), although it is reported by others that the phosphorylation of S15 alone is not sufficient for the inhibition of the binding of p53 to MDM2, however, it is certainly required for trans-activation of p53 (Dumaz and Meek, 1999). S20 phosphorylation plays an important role in the

stabilisation of p53. Replacement of S20 with an alanine (a residue incapable of being phosphorylated) makes p53 highly sensitive to MDM2 mediated degradation (Chehab *et al.*, 1999, Dumaz *et al.*, 2001). There is also involvement of phosphorylation of T18 in p53 stabilization (Chao *et al.*, 2000). Interestingly, the phosphorylation of S15 facilitates subsequent phosphorylation of T18 and S20 (Appella and Anderson, 2001). DNA damage induced phosphorylation of N-terminal residues S15, S33 and S37 also stabilises the binding of p300 (Dumaz and Meek, 1999) and CBP to p53. The FAT (transcriptional factor acetyl-transferase) activity of these proteins acetylate p53 (see Figure 1.4) (Sakaguchi *et al.*, 1998) at residues K370, K371, K372, K381 and K382. This acetylation abrogates the ability for MDM2 to ubiquitylate p53 (Li *et al.*, 2002) and also promotes the DNA-binding activity of p53 (Gu and Roeder, 1997). Other sites of p53 that are acetylated after stress include K120 and K164, via p300/CBP. Mutation of K164 causes a loss of acetylation and abolishes the transcriptional activity of p53 (Tang *et al.*, 2008). However the mutation of K120 to an R120 residue inhibits p53 mediated transcription of pro-apoptotic target genes, such as BAX, but not targets such as p21 and MDM2 (Sykes *et al.*, 2006), suggesting different acetylated sites mediate p53 transcription at different gene promoters.

Other sites phosphorylated in the trans-activation domain in response to stress are S33, S37, S46 and S6 and S9 (Meek and Anderson, 2009) and T81. There are also sites post-translationally modified in the DNA binding domain under unstressed conditions. These include the phosphorylation of S149, T150 and T155. The phosphorylation of S215 is well documented to inhibit the binding of p53 to DNA (Liu *et al.*, 2004).

Phosphorylation of p53 in the C-terminus also occurs after stress, including the phosphorylation of S315 and S392 (Meek and Anderson, 2009). Phosphorylation of S315 stimulates p53 mediated transcription (Blaydes *et al.*, 2001), whereas phosphorylation at S392 stabilizes tetramerization of p53 (Sakaguchi *et al.*, 1997).

Various residues in the MDM2 protein are also targets for post-translational modification (Meek, 2004, Meek and Knippschild, 2003). Phosphorylation of certain residues interrupt the binding of p53 to MDM2 and inhibit MDM2 mediated proteasomal degradation, such as the phosphorylation of S17, Y394 and S395, which are targets of the kinases DNA-PK, c-Abl and ATM proteins respectively (Maya *et al.*, 2001, Meek and Anderson, 2009, Meek and Knippschild, 2003). De-phosphorylation of MDM2 also plays a large part in the interruption of p53 binding to MDM2 (see Figure 1.4). A cluster of serine residues in the central domain of MDM2 are constitutively phosphorylated under unstressed conditions and hypo-phosphorylated after IR (Ionising Radiation). When these phosphorylation sites were substituted from serine to alanine, (mutations that abrogate phosphorylation status) the ability of MDM2 to degrade p53 was reduced or even abolished (Blattner *et al.*, 2002).

Phosphorylation and de-phosphorylation of MDM2 may explain why MDM2 is destabilised after DNA damage by auto-ubiquitylation. Auto-ubiquitylation causes the half life of MDM2 to decrease in cells that have undergone DNA damage. The importance of this destabilisation in p53 activation was shown by an experiment which involved the addition of a proteasomal inhibitor which blocks MDM2 destabilisation (Stommel and Wahl, 2004) and which was used in DNA damaged cells. In the presence of the inhibitor, p53 was incapable of transcriptional activation

in DNA damaged cells (Stommel and Wahl, 2005). To show this was due to MDM2, an inhibitor of the MDM2/p53 interaction, which binds to the p53 binding domain of MDM2, was added and p53 transcriptional activity was rescued.

p53 can be stabilised by the disruption of the p53-MDM2 auto-regulatory loop by inhibition of the interaction of p53 to MDM2 via post-translational modification of both proteins. However the stabilisation of p53 can also be due to, for example, downregulation of *MDM2* expression or regulation of the sub-cellular localisation of p53 and/or MDM2 (Ashcroft *et al.*, 2000). p14^{ARF} (ARF) is one such protein that activates and stabilises p53 (Figure 1.4) and it does this by various mechanisms: it binds directly to MDM2, via the MDM2 C-terminus, causing its degradation (Zhang *et al.*, 1998) or as other authors suggest, sequesters MDM2 to the nucleolus by inhibiting MDM2 nucleolar export allowing nucleo-cytoplasmic p53 activation and stabilisation (Weber *et al.*, 1999). ARF also inhibits the nucleo-cytoplasmic shuttling of the p53-MDM2 complex (Tao and Levine, 1999). Lastly, ARF is also able to inhibit the E3 ligase activity of MDM2 (Honda and Yasuda, 1999).

Two particular studies describe the down-regulation of the *MDM2* gene from two forms of DNA damage. Wu *et al.*, 1997 show that high dose UV irradiation induces *MDM2* repression in a p53 independent manner. Furthermore, the repression of *MDM2* is temporal as *MDM2* is later induced by p53, inhibiting p53 transcriptional activity after a sufficient period of time for DNA repair (Wu and Levine, 1997). Arriola *et al.*, 1999 show that the addition of the topoisomerase inhibitor, Etoposide, inhibits the expression of MDM2 at the mRNA level and therefore there is a decrease in MDM2 protein levels. Etoposide does not induce global repression as

shown by data that show p21 (p21 is a transcriptional target of p53 (el-Deiry *et al.*, 1994, el-Deiry *et al.*, 1993)) expression still exists after treatment of Etoposide.

There are many mechanisms that can stabilize p53 in response to stress and DNA damage and some of these are reviewed in Figure 1.4. The mechanisms by which they occur vary and depend on the type of stress (or DNA damage) that is applied and the strength of the stress. The variety of stresses and mechanisms which stabilise and activate p53 and which ultimately activate the DNA damage pathway, after DNA damage, is reviewed in Section 1.6.

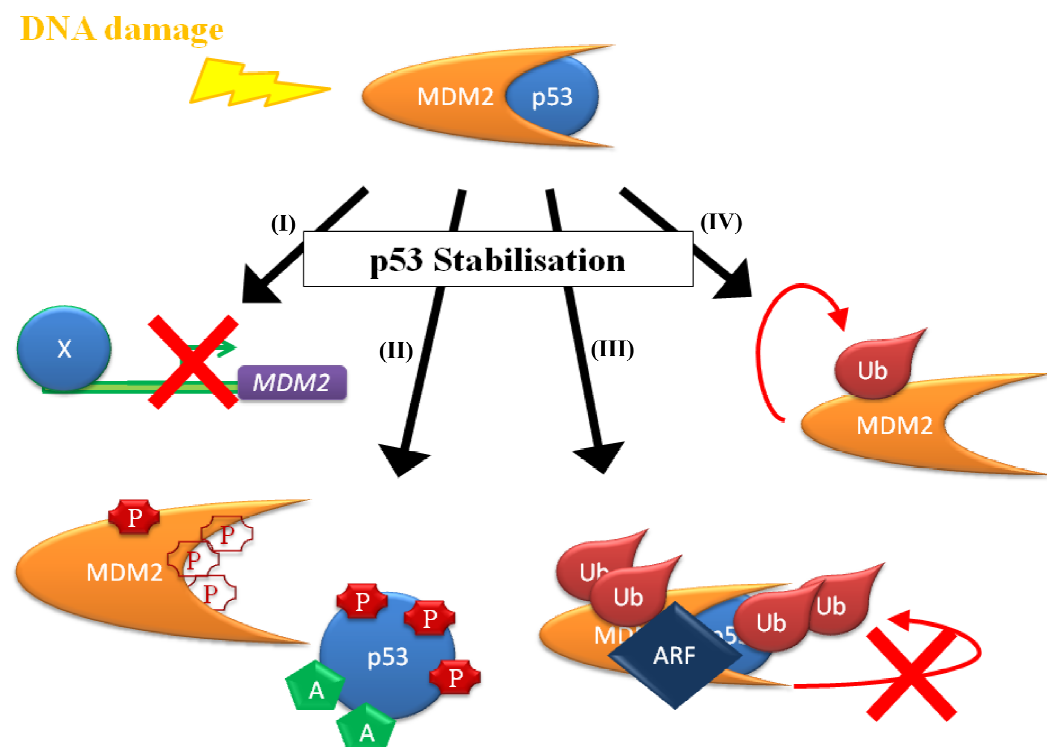


Figure 1.4: The stabilisation of p53 via the disruption of the p53-MDM2 auto-regulatory feedback loop

The p53-MDM2 auto-regulatory feedback is disrupted by various mechanisms, some of which are shown above. This can occur through: (I) the repression of MDM2 expression (II) post-translational modifications (red filled P = Phosphorylation, White filled P = de-phosphorylation, green A = acetylation) which disrupts the interaction of p53 and MDM2 (III) p14ARF binding inhibits MDM2 E3 ligase activity from targeting p53 for degradation and (IV) self mediated auto-ubiquitylation of MDM2.

1.6 Activation of the p53 pathway

There are many types of stress that cause the stabilisation and activation of p53 and the p53 pathway. These include: DNA double strand breaks (DSBs), replication stress, hypoxia and oncogenic activation (see Figure 1.5). Various stress response pathways are described comprehensively in Anderson et al., 2009. For the purposes of this thesis only DNA damage stress, through DSBs and single stranded DNA and replication stress, shall be reviewed. This is because this thesis focuses on G2/M checkpoint arrest, which, is activated by these kinds of DNA damage.

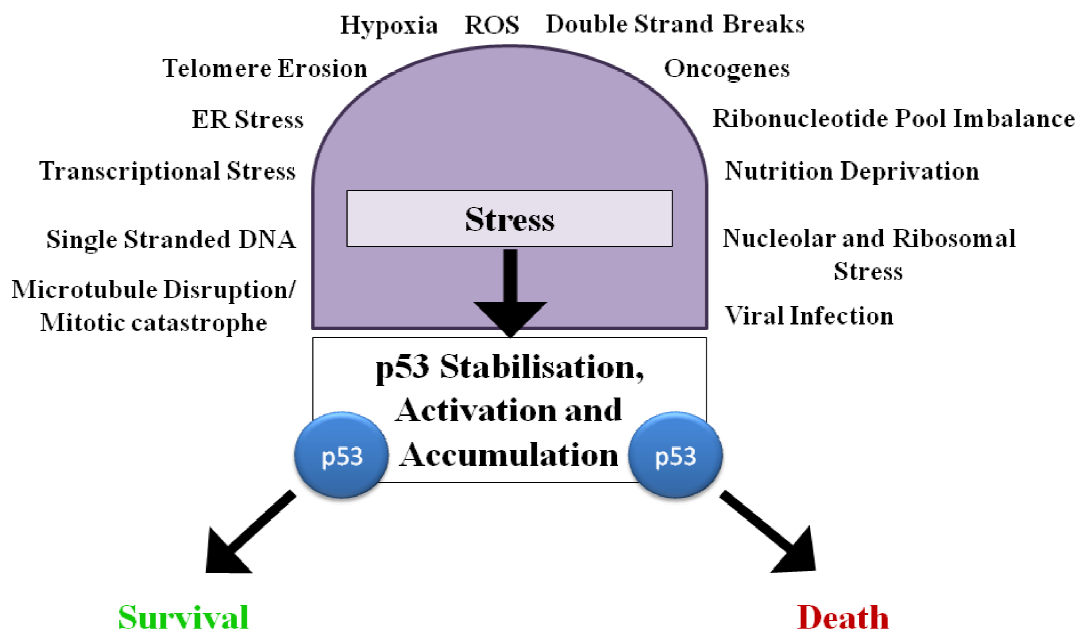


Figure 1.5: Types of stress that activate p53

There are a number of stresses that induce the stabilisation, activation and accumulation of p53 in the cell. Genotoxic stresses include: double strand breaks, single stranded DNA, transcriptional stress, oncogenic activation. Non-genotoxic stresses include Microtubule disruption, ER stress, nucleolar and ribosomal stress, nutrition deprivation, ribonucleotide deprivation.

1.6.1 Double Strand Breaks (DSBs)

The most studied type of genotoxic stress that activates p53 is DNA double strand breaks (DSBs). The key proteins that are activated by DSBs are the MRN complex (Mre11, Nbs1, Rad50) and the protein kinase, ATM (Ataxia- Telangiectasia Mutated) a member of the phosphatidylinositol-3-kinase-like kinase (PIKK) family. When a DSB has occurred, the MRN complex recognizes the DSB and signals to down-stream targets such as ATM (Rupnik *et al.*, 2008). Mre11 binds to DNA and has a local DNA unwinding activity. It is also able to bind Nbs1 and Rad50. Rad50 is able to tether DNA ends together through its dimerization. Nbs1 is responsible for the localisation of the complex into large focal structures and also functions in downstream signalling through ATM (For review see (Rupnik *et al.*, 2008)). Many of these proteins are still being discovered (Lee and Paull, 2007) and therefore the exact mechanism of detection of DSBs is unclear and is an active area of study.

The activation of ATM is not only dependent on the MRN complex but also dimer dissociation of ATM. Reports suggest that auto-phosphorylation and other post-translational modifications of various residues of ATM are important for the activation of ATM. These include the phosphorylation of S1981 in ATM, that causes dimer dissociation and which initiates ATM kinase activity (Bakkenist and Kastan, 2003). Activation is not dependent on direct binding of ATM to DNA strand breaks, but may have resulted from changes in the structure of chromatin (Bakkenist and Kastan, 2003). Although some reports show that S1981 is indispensable for ATM activation, other residues such as S367 and S1893, which were phosphorylated after Ionizing Radiation alongside S1981 are also required for ATM activation. Auto-phosphorylation of all three sites is important in the activation of ATM, as

phosphorylation site mutants were each defective in ATM signalling (Kozlov *et al.*, 2006).

Activated ATM mediates the activation of a variety of downstream proteins, including MDM2, chromatin bound histone H2AX, CHK2 (checkpoint kinase 2) and p53 itself (for review see (Kurz and Lees-Miller, 2004)). Phosphorylation of MDM2 by ATM, occurs at the S395 residue of MDM2 and attenuates MDM2 inhibition of p53 (Maya *et al.*, 2001). This phosphorylation and that of S15 of p53 augment inhibition of the interaction of p53 to MDM2 and its ability to mediate degradation of p53 (Banin *et al.*, 1998). The phosphorylation of CHK2 by ATM at T68 after IR exposure (Ahn *et al.*, 2000, Matsuoka *et al.*, 2000) activates CHK2 and therefore activates CHK2 mediated phosphorylation of several substrates that mediate cell-cycle arrest, DNA repair and apoptosis. For example, CHK2 phosphorylates CDC25C on Ser 216 which inhibits CDC25C function and CDC25C mediated progression through the G2/M checkpoint in the cell cycle (Matsuoka *et al.*, 1998).

ATM activation, after double strand breaks, mediates cell cycle arrest until the cell has repaired the DNA damage. Mutations in the ATM gene are responsible for the rare autosomal recessive disorder ataxia–telangiectasia (A–T), characterised by many phenotypes including defective DNA damage-induced activation of the cell cycle checkpoints at G1, S and G2/M (Chun and Gatti, 2004) and an increased risk of cancer.

Examples of reagents that activate the p53 response by inducing DNA double strand breaks are the DNA damaging agents, Etoposide and Genistein, both of which mediate their effects by inhibiting topoisomerase II. Topoisomerase II is a protein that relaxes the tension in DNA by introducing transient double strand breaks into

the DNA. When topological DNA entanglements occur, for example during transcription and replication, topoisomerase II is able to break the double strand DNA bonds, causing a transient bridge to form, relaxing the tension and then quickly re-ligates the DNA back together. It is therefore an essential protein required in proliferating cells.

Etoposide is a widely available anti-cancer drug and its mechanism of action has been well studied. As described by Burden, *et al.*, 1996, Etoposide mediates its effect by inhibiting topoisomerase II. The inhibitor Etoposide is able to bind to the topoisomerase II protein in such a way that it inhibits the re-ligation of the DNA and therefore increases the number of DNA double strand breaks in the cell. The increase in double strand breaks causes destabilisation of the genome and ultimately disrupts cell viability. Genistein mediates its effects in a similar manner to that of Etoposide (Markovits *et al.*, 1989), however its activation of the p53 pathway is dependent upon ATM, whereas p53 activation by Etoposide is not ATM dependant (Ye *et al.*, 2001). Described by Ye *et al.*, 2001, both agents induce an increase in p53 protein levels as well as the phosphorylation of p53 at S15 however in ATM deficient cells, only Etoposide can induce an increase of p53 and its phosphorylation. This suggests that, even though both agents have a similar mechanism of action, Etoposide activates other downstream targets as well as ATM. An example of which is the ATR (ATM and Rad3 related) protein kinase (Cliby *et al.*, 2002), which is more commonly associated with other types of DNA damage.

Activation of p53 by Etoposide can be induced by direct phosphorylation of p53 via ATM, ATR or both protein kinases and their downstream targets, such as CHK1 and CHK2, which also phosphorylate and activate p53 (Banin *et al.*, 1998, Shieh *et al.*,

2000) (for review see (Bartek and Lukas, 2003)). Furthermore, both PIKK members mediate the degradation of MDM2 under stressed conditions which leads to the disruption of the p53-MDM2 auto-regulatory feedback loop (Stommel and Wahl, 2004).

Clearly all proteins that are mentioned above, from the MRN complex to downstream p53 activators such as CHK1 and CHK2, are important for DSB induced activation of p53.

1.6.2 Single stranded DNA and replication stress

The key protein in detecting other types of DNA damage, such as DNA cross-linking caused by the DNA damaging agent Cisplatin, is ATR. Cisplatin has been studied in depth over the years, and its mechanism of action is thought to cause intra-strand and inter-strand cross links. Subsequently DNA adducts are formed by Cisplatin which links strands of DNA together and in doing so contorts and bends the DNA. However as a result of these effects the transcription machinery cannot transcribe the DNA. ATR is activated in response to the blocked transcription and replication caused by the DNA adducts produced by Cisplatin and other bulky DNA lesions produced by agents such as 5-fluorouracil and UV light (Ljungman and Lane, 2004). As reviewed in Ljungman *et al.*, 2004, these types of DNA damage cause the RNA polymerase II (Pol II) complex to stall and leads to activation of Pol II dependent transcription coupled nucleotide excision repair (CT-NER). They also induce the p53 pathway (Ljungman *et al.*, 1999).

ATR is activated in response to single stranded DNA (ssDNA). ssDNA occurs under non stressed conditions, for example, during DNA replication and in stressed conditions where DNA damage has occurred (Shechter *et al.*, 2004). In response to single stranded DNA, replication protein A (RPA) binds and coats the length of the single stranded DNA. This attracts two key complexes: the ATRIP-ATR and 9-1-1 complex. The ATR binding protein, ATRIP, is bound to ATR and is itself phosphorylated by ATR and regulates ATR expression (Cortez *et al.*, 2001). ATR and ATRIP both localize to the nucleus after DNA damage or inhibition of replication. Interestingly, the deletion of ATR abolished cell cycle checkpoint activation by DNA damage (Cortez *et al.*, 2001).

The 9-1-1 complex consists of the proteins: RAD9, RAD1 and HUS1 which form a heterotrimeric ring. The recruitment of the RAD17 RFC protein mediates the binding of the 9-1-1 protein onto the DNA (Bermudez *et al.*, 2003). The recruitment of a second protein, TopBP1, to the 9-1-1 complex (in particular RAD9) is necessary for the binding of the ATR-ATRIP complex to TopBP1 (Lee *et al.*, 2007) and TopBP1 in turn activates ATR through its ATR activating domain (Kumagai *et al.*, 2006). Once activated, ATR phosphorylates downstream targets such as CHK1, which is presented to ATR by the protein, Claspin (Kumagai *et al.*, 2004). The activation of downstream targets of ATR slow down origin firing and induce cell cycle arrest giving the cell time to repair the DNA damage. Once repaired ATR and its downstream targets mediate the stabilization and the resumption of stalled replication forks (for review see (Cimprich and Cortez, 2008). Shechter *et al.*, 2004, suggest that in the event of DNA damage, the stalled fork results in accumulation of RPA at sites of single stranded DNA and accumulation of ATR–ATRIP complexes.

Alongside Claspin and 9-1-1, ATR initiates the checkpoint block (Shechter *et al.*, 2004).

Although CHK1 activates p53, by phosphorylating p53 (Shieh *et al.*, 2000), ATR also directly phosphorylates and activates p53 (Tibbetts *et al.*, 1999). After DNA damage, ATR phosphorylates p53 at S15 and S37 (Tibbetts *et al.*, 1999). The activation of p53 by CHK1 leads to downstream effects such as cellular senescence and apoptosis. Other proteins are also activated, such as BRCA1, H2AX and E2F and are reviewed in detail in Ljungman *et al.*, 2004.

The activation of p53 by DNA damage, such as DSBs or replication stress, is very complex and the route by which p53 is activated depends on the type of stress and the length of time the stress has been applied. It is thought that the ATR pathway activates p53 after a longer period of time compared to that of ATM presumably due to the slowing of origin firing caused by ATR and the attempt of ATR to rescue the cell by repairing DNA damage through the NER pathway. ATM and ATR are not the only proteins involved in the DNA damage pathway and other proteins include DNA-PK, another PIKK member, however, in their absence, the response to DNA damage response is abrogated.

1.6.3 The p53 pathway

There is a variety of mechanisms by which p53 is activated and these are dependent upon the type of stress stimulus. The ATM and ATR pathways play critical roles in the induction of p53 in response to DNA damage. However, the activation of p53 itself is responsible for the regulation of many genes whose products mediate the

cellular outcomes of p53 induction, as shown in Table 1.1. One of the most well known and comprehensively studied is p21 (also known as WAF1 and CIP1), which is required for cell cycle arrest (for reviews see (Shu *et al.*, 2007, Vousden and Lu, 2002)). Others genes that are regulated by p53 are involved in apoptosis, tumour suppression, inhibition of angiogenesis and auto-regulation of p53 (Table 1.1).

Protein/Gene	Function/Role	Trans-activation (T) or Repression (R) by p53	Reference(s)
p21 (CDKN1)	Checkpoint Arrest	T	(el-Deiry <i>et al.</i> , 1993, el-Deiry <i>et al.</i> , 1995, Saramaki <i>et al.</i> , 2006)
GADD45	Checkpoint Arrest and DNA Repair	T	(Kastan <i>et al.</i> , 1992)
14-3-3	Checkpoint Arrest	T	(Hermeking <i>et al.</i> , 1997)
Reprimo	Checkpoint Arrest	T	(Ohki <i>et al.</i> , 2000)
CDC25C	Progression of Mitosis	R	(St Clair and Manfredi, 2006)
MAD1	Progression of Mitosis	R	(Chun and Jin, 2003)
MDM2	Down-regulation of p53	T	(Wu <i>et al.</i> , 1993, Zauberman <i>et al.</i> , 1995)
p53 (TP53)	Various Cellular Outcome Mediator	T	(Benoit <i>et al.</i> , 2000)
Survivin	Inhibitor of Apoptosis	R	(Hoffman <i>et al.</i> , 2002)
BAD	Mediates Apoptosis	T	(Jiang <i>et al.</i> , 2006)
BAX	Mediates Apoptosis	T	(Miyashita and Reed, 1995, Thornborrow <i>et al.</i> , 2002)
PUMA	Mediates Apoptosis	T	(Nakano and Vousden, 2001)
PTEN	Mediates Apoptosis	T	(Stambolic <i>et al.</i> , 2001)
PTGF	Inhibits Tumour Cell Growth	T	(Tan <i>et al.</i> , 2000)
KAI1	Inhibitor of Tumour Metastasis	T	(Mashimo <i>et al.</i> , 1998)
XPC	DNA Repair	T	(Adimoolam and Ford, 2002)
DDB2/p48	DNA Repair	T	(Hwang <i>et al.</i> , 1999, Tan and Chu, 2002)

Table 1.1: Genes transcriptionally regulated by p53

This table comprises a list of some of the genes that are transcriptionally regulated by p53. The table shows the protein (gene) that is activated or repressed, its function or role in the cell, whether its expression is repressed or activated by p53 and its corresponding reference(s).

The main fact that p53 can activate the expression of genes involved in different cellular pathways suggests that it is involved in many cellular processes that occur after DNA damage or other genotoxic and non-genotoxic stresses. These are reviewed in the next Section.

1.6.4 Cellular outcomes of p53 activation

The two main outcomes of p53 activation are cell cycle arrest or programmed cell death (apoptosis). The p53 transcribed genes and proteins that are responsible for these outcomes can be subdivided into the following: DNA repair, autophagy and cell cycle arrest, differentiation, senescence and apoptosis. The type of response is dependent on the level of DNA damage (for review see (Helton and Chen, 2007)). Given that p53 regulates the expression of literally hundreds of genes, studied extensively in Riley *et al.*, 2008, the following discussion will focus on those that are relevant to the work described in this thesis.

1.6.4.1 The cell cycle and its arrest

When the cell has undergone stress and DNA damage has occurred the cell can arrest this cycle at two main checkpoints: G1 and G2/M checkpoint. Notably the cell can also arrest in S phase. These checkpoints are beneficial in the sense that the cell can inhibit the cycle before any mutation can be taken forward into a daughter cell (for review see (Vermeulen *et al.*, 2003)). Cancer cells overexpress oncogenes that can override the checkpoint allowing the cell to proliferate unrestrained and carry forward mutations that may have occurred, into daughter cells.

The main proteins that regulate the cell cycle are the Cyclin Dependent Kinases (CDKs) and Cyclins. CDKs 2 and 4 are required for G1 phase alongside Cyclins D1, D2 and D3. CDK2 alongside Cyclin A is required for S phase and CDK1 with Cyclin B is necessary for mitosis. However, arguably the most important CDKs and Cyclins in the cell cycle are those that regulate the two checkpoints. CDK2/Cyclin E regulates progression of the cell cycle into S phase (G1 checkpoint). Overexpression of Cyclin E activates the CDK2/Cyclin E kinase activity and results in a shorter G1 phase (Ohtsubo *et al.*, 1995). The second important checkpoint proteins are CDK1 (*cdc2*) and Cyclin A/B. Knockout of Cyclin A results in premature entry into mitosis (Walker and Maller, 1991).

Under stressed conditions CDK2/Cyclin E is regulated by the p21 protein. p21, is a protein that is highly induced by p53 via a sequence specific binding site identified in the promoter region of the p21 gene (el-Deiry *et al.*, 1993), as discussed previously. Other studies carried out at the same time identified the p21 protein as a potent inhibitor of G1 phase Cyclin-Dependent Kinases (CDKs) which are required for the progression of the cell cycle (Harper *et al.*, 1993, Xiong *et al.*, 1993). Notably the increase of p53 after DNA damage was concomitant with increases in p21 and this increase was induced in wild type p53 containing cells after DNA damage and could not occur independently of p53 suggesting that p53 is indeed responsible for the increase in p21 (el-Deiry *et al.*, 1994). Furthermore, p21 was found bound to Cyclin E containing complexes which resulted in a decrease in the CDK kinase activity (el-Deiry *et al.*, 1994). All together these data show that via the induction of p21, p53 can mediate a G1/S checkpoint arrest in the presence of DNA damage.

As a regulator of the G1/S checkpoint, overexpression of p21 results in a profound reduction of cells in S phase (el-Deiry *et al.*, 1993). In a p21 null cell line G1 arrest mediated by DNA damage arrest is abolished (Waldman *et al.*, 1995). The p21 protein is responsible for the inhibition of CDK2/Cyclin E complex (and others), and inhibits it from phosphorylating and inactivating the stress induced Rb (retinoblastoma) protein. Phosphorylated Rb cannot then bind to and inhibit the transcription factor E2F (Weinberg, 1995, Weinberg and Denning, 2002). Another route by which p21 causes growth arrest is by binding to E2F which inhibits transcription at the Cyclin A promoter, which suggests that p21 can also have a role to play in the transcription of S phase genes (Delavaine and La Thangue, 1999). Other reports suggest that p21 also functions to bind to and inhibit PCNA (Proliferating Cell Nuclear Antigen) which is a dual role protein that is involved mainly in DNA replication but also DNA repair (Waga *et al.*, 1994). Interestingly, p21 inhibits only the DNA replication activity and not the NER repair pathway activity of PCNA (Shivji *et al.*, 1994).

Another protein that is involved in cell cycle arrest is GADD45A (Growth Arrest and DNA Damage inducible factor), which is a protein induced after DNA damage (Hollander *et al.*, 1993). GADD45A is found to bind to CDK1 (*cdc2*) and inhibits the activity of the CDK1/Cyclin B complex, needed for progression into M phase (Zhan *et al.*, 1999). Zhan *et al.*, 1999, show that the addition of GADD45A did this by inhibiting the association of Cyclin B to CDK1. Kastan *et al.*, 1992 show that GADD45A is a p53 regulated gene, as, in cells that are p53-null, there was no increase in GADD45A. Furthermore mutant p53 could not cause an induction of GADD45A transcription through a sequence specific binding site (Kastan *et al.*, 1992). These authors identify a p53 specific binding site in the GADD45A gene at

intron 3. Taken together these data show that GADD45A inhibition of CDK1/Cyclin B and therefore GADD45A mediated cell cycle arrest is dependent upon p53.

Other proteins that are regulated during the cell cycle checkpoint, in particular the G2/M checkpoint and which are regulated by p53, include 14-3-3 and CDC25C. 14-3-3 is expressed by p53 upon DNA damage. Studies show that the 14-3-3 σ isoform is regulated by p53 through a p53 responsive element in the promoter and was shown to be overexpressed after IR induced DNA damage (Hermeking *et al.*, 1997). The overexpression of 14-3-3 induced a G2/M checkpoint arrest. 14-3-3 is responsible for the cellular localisation of its substrates to various regions of the cell. It is also involved in the activation and repression of the enzymatic activity or function of proteins as well as being involved in the prevention of degradation of some of its protein substrates (Hermeking and Benzinger, 2006). It has many roles in checkpoint such as binding phosphorylated CDC25C causing its cytoplasmic sequestration, therefore 14-3-3 is an important p53 regulated protein required for p53 mediated G2/M arrest.

Another interesting target of p53 is the phosphatase, CDC25C (Cell Division Cycle 25 homolog C), which is highly involved in the G2/M checkpoint. This particular protein is required for the progression of mitosis and is regulated by many proteins such as CHK1 and CHK2 (as reviewed in (Hoffmann, 2000)). p53 has also been found recently to regulate CDC25C in cooperation with another as yet unknown protein. Mutation of the sequence specific p53 binding site abolishes repression of CDC25C by p53 (St Clair *et al.*, 2004, St Clair and Manfredi, 2006). The study by St Clair *et al.*, 2004, was one of the first that demonstrated that repression by p53 can involve direct binding of p53 to sequence specific DNA (p53 Responsive Elements).

Incidentally, the first gene found to be repressed by p53 at the transcriptional level was Survivin (Hoffman *et al.*, 2002) and is discussed in Section 1.6.4.4.

The CDC25C protein is important in the cell cycle as proved by the data that its inhibition, through microinjection of antibodies, blocks entry into mitosis. Furthermore, the introduction of CDC25C, into temperature sensitive CDC25C mutant cells rescued the inhibition of entry into mitosis by CDC25C (Millar *et al.*, 1991). The main role of CDC25C in the cell is the dephosphorylation of CDK1 at T14 and T15. Mutation of the catalytic site of CDC25C abolishes the phosphatase activity of CDC25 (Gautier *et al.*, 1991). These sites are phosphorylated by Wee1 and Myt1 in S phase which inhibit the CDK1/Cyclin B complex from entering the nucleus via the sequestration of the complex by 14-3-3 in the cytoplasm (for review see (Hermeking and Benzinger, 2006)). Evidently, dephosphorylation of the CDK1/Cyclin complex allows progression of the cell into mitosis.

The phosphorylation of residue S216 of CDC25C is of particular importance to the sequestration of CDC25C by 14-3-3 in the cytoplasm. Mutation of this residue abrogates 14-3-3 binding and overexpression of this mutant resulted in an abrogation of G2 delay induced by DNA damage (Peng *et al.*, 1997). Studies of the interaction between CDC25C and 14-3-3 show that the binding of 14-3-3 covers the NLS of CDC25C, which causes cytoplasmic localisation (Kumagai and Dunphy, 1999, Kumagai *et al.*, 1998). CDC25C is inactive for the majority of the cell cycle until mitosis, as shown by the fact that CDC25C phosphatase activity is undetectable during the other three phases. Activation of CDC25C occurs after the dephosphorylation of CDC25C at residues 216 and 287 (Donzelli and Draetta, 2003). After DNA damage however, the CDC25C protein is phosphorylated by

CHK1 and CHK2, at certain individual sites (residue 216 by CHK1/2), therefore causing G2/M cell cycle arrest (Chaturvedi *et al.*, 1999, Furnari *et al.*, 1997, Peng *et al.*, 1997, Sanchez *et al.*, 1997). As previously stated the CDC25C protein is a target of p53 and is repressed by p53 via a p53 responsive element located in its promoter (St Clair and Manfredi, 2006). Taking these data together suggest that p53 and 14-3-3 act co-ordinately to repress CDC25C after DNA damage to cause G2M checkpoint arrest.

There are multiple p53 targeted proteins that are involved in cell cycle and its arrest, only some of which have been reviewed in this thesis. However, through the activation or repression of these proteins, p53 enables cell cycle checkpoint arrests after DNA damage and stress and allows the cell to halt its cycle until the DNA damage has been repaired. This cellular outcome of p53 is very important, as without it the cell would proliferate with the mutations arising from DNA damage and DNA damage would be carried into daughter cells where they may contribute to tumourigenesis.

1.6.4.2 DNA repair

When the cell cycle has arrested due to DNA damage the cell endeavours to repair the damage through different types of pathways depending on the type of stress and lesion that has occurred. These pathways include the NER (nucleotide excision repair) pathway, which can be divided into the TCR (transcription coupled repair) and GGR (global genome repair). Others are the BER (base excision repair), MMR (mismatch repair pathway), HR (homologous recombination), NHEJ (non-

homologous end joining) and TLS (translesion synthesis) (for review see (Helton and Chen, 2007)).

Markedly, p53 has been found to play a role in all of these pathways by activating or repressing genes either involved in these processes or directly through interaction with other proteins at the repair site. The NER pathway is one of particular interest in this thesis due to the fact that the drug Cisplatin causes induction of this pathway. The NER-TCR and NER-GGR pathways are distinct from each other in the way they detect DNA damage. The NER-TCR pathway detects damage through the blockage of RNA Pol II elongation and therefore only detects damage in RNA Pol II transcribed genes, which makes up only a part of the genome. On the other hand, the GGR pathway detects damage through proteins that detect DNA damage including DDB1 (p127), DDB2 (p48/XPE) and the XPC/hHR23B complex (Costa *et al.*, 2003) in other parts of the DNA.

Targets of p53 involved the GGR pathway include XPC and DDB2 (p48). These genes are targets of p53 activated expression (Helton and Chen, 2007). It has been shown that p53 is required for the NER of CPDs (cyclobutane pyrimidine dimers), as p53-null cells cannot repair CPDs. Repair of CPDs is independent of the NER-TCR pathway (Adimoolam and Ford, 2002). These authors show that the XPC gene is p53 regulated and is not expressed in p53-null cells. XPC induction also occurs after DNA damage in a p53 dependent manner. Likewise, p48 is also p53 regulated. Data show that in p53-null cells p48 was not induced by DNA damage, however in p53 wild type cells p48 levels increased concomitantly with increases in p53 after DNA damage. Furthermore the authors show that a mutant p48 cell line, is defective in

GGR of CPDs and therefore suggest that GGR is dependent on both p48 and p53 (Hwang *et al.*, 1999).

Less is known about targets of p53 in the TCR pathway however data show that p53 has been found to bind to proteins involved in the NER pathway including XPB and XPD. This suggest that p53 modulates the DNA repair pathway directly as well as expression of proteins involved in the DNA repair pathway (Wang *et al.*, 1995).

DNA repair pathways other than NER include HR and NHEJ. The dependence of these pathways on p53 is largely unknown however studies show that mice that express mutant p53, ATM or GADD45A have a higher incidence of HR (Bishop *et al.*, 2003) suggesting p53 negatively regulates HR. However, which pathway is utilized depends on the type of stress, for example Cisplatin induced DNA adducts are repaired via the NER pathway whereas Etoposide induced DNA double strand breaks are thought to be repaired by HR and NHEJ, if the cell allows for DSB repair at all due to the potential of deletion or translocation of DNA that could affect genomic stability. DNA repair is nevertheless a major cellular outcome of the activation of p53 and is an option to assist the cell in overcoming DNA damage.

1.6.4.3 Senescence

Senescence is another outcome of p53 activation and is a term used to describe cells that no longer replicate, due to shortened telomeres or non-telomeric signals such as DNA damage and oncogenic signals (Dimri, 2005). Senescence limits the replicative capacity of cells and therefore inhibits abnormal proliferation. The erosion of telomeres activates the DNA damage response similar to that of double

strand breaks (d'Adda di Fagagna *et al.*, 2003). d'Adda di Fagagna *et al.*, 2003 show that telomeric induced senescent cells have activated CHK1 and CHK2 as well as high levels of DNA repair proteins. The activation of these kinases induces p53, and in turn, the activation and accumulation of p21, which inhibits CDKs. This inhibition of CDKs consecutively causes the levels of hypo-phosphorylated Rb (a tumour suppressor) to increase causing further cell cycle arrest and senescence (Dimri, 2005). Non-telomeric signals, on the other hand, activate both the CHK1/2-p53-p21-Rb pathway and p16^{INK4a}-Rb pathway (p16^{INK4a} is a product of the alternate reading frame of *CDKN2A*, the other product being p14^{ARF}) (for review see (Dimri, 2005)) to cause permanent cell cycle arrest. For the purposes of this thesis, the discussion of this outcome of p53 activation has been kept short.

1.6.4.4 Apoptosis

Apoptosis is one of the key main outcomes of p53 activation and allows the cell to undergo programmed cell death when DNA damage cannot be repaired. Apoptotic cells, from the morphological point of view, are characteristically round and tend to have condensed cytoplasm and fragmented nuclei (Elmore, 2007). Apoptotic cells degrade their organelles and other cellular contents leading to the formation of “membrane blebs”; these are then recognised and digested or phagocytosed by macrophages and other surrounding cells. In comparison to cells that have undergone necrosis, there is no release of the cellular contents into the surrounding tissue and there is no activation of an inflammatory response that occurs in response to necrosis (Elmore, 2007). From the biochemical view, cells that are undergoing apoptosis show signs of protein cleavage, protein cross-linking, DNA breakdown

and phagocytic recognition. These collectively cause the distinctive apoptotic pathology that has been described above.

There are two main apoptosis pathways: the extrinsic (death receptor pathway) and the intrinsic (mitochondrial pathway) (Elmore, 2007). The death receptor pathway consists of a ligand, for example TNF (Tumour Necrosis Factor), that binds to a receptor (TNF Receptor) initiating the binding of other proteins, such as TRADD and FADD to the internal component of the receptor. Another example is the Fas ligand which binds to the Fas receptor and then mediates the binding of FADD (Kischkel *et al.*, 1995). The ligand, receptor and associated proteins together form the DISC (Death Induced Signalling Complex). Pro-Caspase 8 (initiator Caspase, also known as FLICE) is recruited to the DISC and activated through a series of proteolytic cleavage steps. Caspase 8 in turn activates the effector Caspase 3 (Andersen *et al.*, 2005, Elmore, 2007).

The intrinsic pathway involves the mitochondrial release of Cytochrome C (which mediates apoptosis in a Caspase dependent manner), apoptosis-inducing factor (AIF) and endonuclease G (which mediate apoptosis in a Caspase independent manner) (Saelens *et al.*, 2004) and is activated by apoptotic stimuli such as chemotherapy and UV. These stimuli activate pro-apoptotic proteins, such as BAX, which mediate changes in the inner mitochondrial membrane and which result in the opening of the mitochondrial permeability transition (MPT) pore and the release of Cytochrome C (Jurgensmeier *et al.*, 1998, Pastorino *et al.*, 1998). Other pro-apoptotic proteins include BAK, BAD and BID. The mediation of the release of Cytochrome C by these proteins allows Cytochrome C to bind to and activate Apaf1 and Caspase 9 (an

initiator Caspase), forming the apoptosome. The apoptosome consequently cleaves pro-Caspase 3 into its activated form (Saelens *et al.*, 2004).

Both the intrinsic and extrinsic pathways activate effector Caspase 3 which executes the final pathway of apoptosis by cleaving various substrates including cytokeratins and PARP and activates both cytoplasmic endonucleases (which degrade nuclear material) and proteases (which degrade nuclear and cytoskeletal proteins).

Apoptosis is one of the key outcomes of p53 activation. p53 regulates apoptosis through many mechanisms and one of these key mechanisms includes the activation of expression of the pro-apoptotic Bcl-2 members. One example, BAX (Bcl-2 Associated X protein), binds to the Bcl-2 protein. The binding of these two proteins inhibits BAX pro-apoptotic activity. Under stressed conditions, BAX dissociates from Bcl-2 and is relocated from the cytosol to the mitochondrial membrane. Mutated BAX protein is unable to localise to the mitochondria and this reduces the cell death activity of BAX (Wolter *et al.*, 1997). p53 induces BAX expression (Table 1.1) through a sequence specific p53 DNA binding site in the promoter of the gene and mutation of this site abolishes p53 responsiveness of reporter gene plasmids suggesting that BAX induction of apoptosis is dependent upon p53 (Miyashita and Reed, 1995).

BAD is another pro-apoptotic protein and member of the Bcl-2 family, which is p53 regulated (Table 1.1). BAD mediates apoptosis through binding to Bcl-2 and in doing so displacing BAX, allowing BAX to promote apoptosis (Yang *et al.*, 1995). p53 induces expression of BAD (Bcl-2 Antagonist of cell Death) (Jiang *et al.*, 2006). These authors demonstrate that p53 induces the expression of BAD through a sequence specific p53 DNA binding site, after DNA damage. Furthermore, the

increase in BAD increases the binding of p53 to BAD in the cytoplasm, inhibiting p53 from entering the nucleus and which ultimately decreases the expression of BAD through a negative feedback loop. BAD instead directs p53 to the mitochondria to induce Cytochrome C release. Downregulation of BAD by siRNA reduces mitochondrial p53 and increases the resistance to the chemotherapy agent, Etoposide (Jiang *et al.*, 2006).

Proteins that inhibit apoptosis and are p53 regulated include the IAPs (Inhibitors of Apoptosis), such as Survivin. Survivin binds to and inhibits the Caspase activity of Caspase 3 (Tamm *et al.*, 1998). Survivin has a role to play in spindle formation as the disruption of the Survivin-microtubule complex induces Caspase 3 activity and apoptosis (Li *et al.*, 1998). It is also a cell cycle regulated protein and levels of Survivin increase and are at their highest during mitosis (Li *et al.*, 1998). Loss of Survivin induced apoptosis and this was heightened in p21-null cells, indicating a role for Survivin in the protection of the p21-centrosome complex which is required for progression of mitotic progression (Li *et al.*, 1999). Taking these data together suggests that the down-regulation of Survivin would be beneficial to a cell that is required to undergo apoptosis. Work carried out by Hoffman *et al.*, 2002 show that p53 binds to the *Survivin* gene promoter at a p53 responsive element and overexpression of p53 down-regulates Survivin. The downregulation of Survivin by p53 is not p21 dependent and furthermore overexpression of Survivin in cells that are dependent on p53 mediated cell death inhibits apoptosis induced by UV (Hoffman *et al.*, 2002). Taken together, these data show that the repression of Survivin by p53 is a key mechanism in p53 mediated apoptosis.

Examples of proteins that are involved in the extrinsic pathway of apoptosis and that are p53 regulated is the Fas (also known as APO-1 or CD95) Receptor (Muller *et al.*, 1998) and Fas ligand. These authors show that only wild type p53 and not mutant p53 up-regulates the Fas Receptor and expression of wild type p53 into a p53-null cell background re-establishes CD95 mediated apoptosis. Furthermore these authors show that the Fas ligand is up-regulated in response to DNA damage, albeit this up-regulation is not p53 dependent as this up-regulation occurs in p53-null and p53 mutant cell lines. However the authors show that p53 activates CD95 expression through p53 responsive elements in the promoter and another in the first intron of *CD95* and mutant p53 could not induce apoptosis through expression of CD95 (Muller *et al.*, 1998).

1.7 Gene regulation by p53

As discussed in Section 1.4.2, p53 binds to DNA via a sequence specific DNA site or p53 responsive element from where it then trans-activates or represses transcription of the gene. However there are other important transcription factors that regulate genes at the promoter other than p53. For example, the CCAAT box is the DNA recognition site for NF-Y (CBF or CP1) (Mantovani, 1999); the TATA box is commonly known to be the site at which transcription factor TFIID binds to and where the Pol II transcription machinery assembles. The TFIID complex consists of many proteins including TAF(II)250, TAFs and TBP (TATA Binding Protein), which is the protein required for TFIID and other TAFs to bind to the TATA box (Burley and Roeder, 1996, Hernandez, 1993). These sites are also sites that proteins such as p53 can act through, such as the binding of p53 to TBP-TATA box complex

or p53 binding to the NF-Y-CCAAT box complex (Martin *et al.*, 1993, Truant *et al.*, 1993). The Sections below aim to discuss the different elements in the activation and repression of gene expression and the role p53 has to play in their regulation.

1.7.1 TATA Box, TBP and p53

TBP is a protein that is essential for transcription (Kim and Iyer, 2004). It interacts with and is required by all three types of RNA Polymerase (I-III) for transcription (Cormack and Struhl, 1992, Hernandez, 1993) and therefore is recruited to all three basal promoter elements. Interestingly, RNA Pol I promoters do not contain a TATA box, whereas most RNA Pol II and III do. TBP in this case can bind to TATA-less promoters through protein-protein interactions (Hernandez, 1993). Data show that TATA-less promoter transcription requires the TFIID complex (Pugh and Tjian, 1991) and accordingly, RNA Pol II TATA-less promoters can bind TBP through an initiator sequence (Hernandez, 1993). Kim *et al.*, 2004, show that TBP can bind to TATA-less promoters, show that TBP has a higher affinity for Pol III promoters. Furthermore, data show that the transcription of these genes throughout the genome are proportional to the occupancy of TBP on their promoters, as alterations in the expression of these genes are concomitant with alterations in TBP recruitment to their promoters (Kim and Iyer, 2004), which is regardless of the presence or absence of a TATA box.

The interaction of TBP-TATA box complex and p53 has been well documented (Chen *et al.*, 1993, Martin *et al.*, 1993, Seto *et al.*, 1992, Truant *et al.*, 1993, Xing *et al.*, 2001). The three earlier publications show that p53 binds to TBP and that the

complex can bind directly to the TATA box. Truant *et al.*, 1993 show that mutation of residue R175 (which lies in the DNA binding domain of p53) to H175, reduced p53 binding to TBP and consequently reduced transcription. The interaction between p53 and TBP was further confirmed by Chen *et al.*, 1993 whom show that p53 and TBP act co-ordinately to activate gene expression. Furthermore, other reports (Seto *et al.*, 1992) suggest that p53 binding to TBP can also cause gene repression. p53 can also regulate gene expression by stimulating the assembly of other TAFs onto the complex (Xing *et al.*, 2001).

Interestingly, p53 cooperates with TBP (or TFIID) in binding to DNA which contains both a specific p53 responsive element and a TATA box, and both stimulate p53 binding to the DNA promoter which contains only a p53 response element (Chen *et al.*, 1993). However both do not collaborate in the binding of promoters with only a TATA box and data show that p53 in fact inhibits TBP binding to the TATA box in the absence of a p53 responsive element (Chen *et al.*, 1993).

It is unclear whether the outcome of gene expression by p53 (whether the gene is activated or repressed), is TATA box dependent, as p53 also represses TATA-less promoters (Gopalkrishnan *et al.*, 1998). However, what can be concluded is that the involvement of p53 with the TBP protein (and TATA box) can regulate gene expression.

1.7.2 CDE/CHR (Cell Cycle Regulatory) Element

The cell cycle-dependent element (CDE) and the cell cycle genes homology region (CHR) control the transcription of genes that are expressed in G2 phase and in

mitosis, for example CDC25C (Lucibello *et al.*, 1995, St Clair *et al.*, 2004), cdc2 (Zwicker *et al.*, 1995b) and PLK1 (Uchiumi *et al.*, 1997). The elements' consensus sequence consists of the following: the CDE, N N T/G G/C G C G G N A/G (N represents any nucleotide (Zwicker *et al.*, 1995b)) or CGGCGCC (Muller and Engeland, 2010) and the CHR, N G/A T/C T T G A A N N (Zwicker *et al.*, 1995b) or TTTGAA (Muller and Engeland, 2010). The CDE was first described in the promoter of CDC25C (Lucibello *et al.*, 1995) and is always accompanied by a second conserved site, termed CHR (Zwicker *et al.*, 1995b). Interestingly, the CDE/CHR element is usually found in promoters that contain multiple CCAAT boxes and that lack a TATA box (Muller and Engeland, 2010), such as that of CDC25C (Korner *et al.*, 1997). Notably, there is some similarity between CDE and the E2F binding site when comparing their consensus sequences and one way of distinguishing the two is that the E2F binding site is not found next to a CHR element. However, it is not clear how E2F is able to distinguish between the E2F binding site and the CDE element or what specificity E2F has for the E2F binding site or CDE as data suggests that E2F not only binds its consensus site but also the CDE element (Muller and Engeland, 2010).

The function of the CDE/CHR element is to regulate cell cycle genes, specifically those that express proteins required for G2/M by repressing the gene up until the G2 and mitosis phases of the cell cycle. This function was shown by Lucibello *et al.*, 1995 whose data identify that mutation of CDE in the CDC25C promoter causes the activation of gene expression throughout the cell cycle, specifically the G1 and S phases of the cell cycle. Furthermore, mutation of the CHR also deregulates CDC25C and other proteins such as Cyclin A and cdc2 in such a way that they are continually expressed throughout the cell cycle (Zwicker *et al.*, 1995b). Another

example of cell cycle regulated expression of G2/M phase genes through the CDE/CHR element is *PLK1*. As like *CDC25C* mutation of either the CDE or CHR, abrogates G1 and S repression of *PLK1* and levels of *PLK1* expression are constitutively high (Uchiumi *et al.*, 1997).

Cell cycle regulated genes, in particular those involved in G2 and M phase, are repressed by p53 and one of the mechanisms by which p53 can do this is through the CDE/CHR element (St Clair *et al.*, 2004). Repression by p21 is also mediated through the CDE/CHR element (Zhu *et al.*, 2002). These represent other mechanisms for p53 (and p21) dependent cell cycle arrest in response to DNA damage and other stresses. St Clair *et al.*, 2004 characterise the extent to which p53 represses the *CDC25C* gene and propose that there are two mechanisms by which p53 represses *CDC25C*: through a p53 binding site and through the CDE/CHR element. Interestingly, p53 represses expression of a Luciferase reporter gene, not only through a p53 responsive element but also through the CDE/CHR element and mutation of this element almost abolishes repression of the reporter gene. However titring of p21 into the system (rather than p53) does repress expression through the CDE/CHR element, suggesting that the induction of p21 may be how p53 represses *CDC25C*. Mutation of the CDE/CHR element completely abolishes repression of the reporter gene by p21. Further to this, these authors show that mutation of the CDE/CHR element only affects repression and not binding of p53 to DNA. They also take into consideration the CCAAT boxes in the *CDC25C* promoter. Whilst p53 represses expression through the CCAAT boxes alone, repression does not occur at physiological levels of p53, shown by experiments using a cell line that induces p53 expression on the withdrawal of Tetracyclin. Lastly, they show that p53 is not bound to the CCAAT box. A later publication by this group, as already described in this

introduction, verifies that there is also a strong involvement for the p53 RE in repression of CDC25C by p53 as well as the CDE/CHR element (St Clair and Manfredi, 2006).

Other reports imply that DNA damage induced down-regulation of CDK1 (*cdc2*) at the G2/M checkpoint, is dependent upon p53 mediated repression through the CDE/CHR element (Badie *et al.*, 2000).

Conclusively, the repression of the repressive activities of the CDE/CHR element by p53 is clearly another mechanism by which p53 mediates G2/M checkpoint arrest induced by DNA damage and other stresses.

1.7.3 CCAAT Box, NF-Y and p53

Many proteins bind to the CCAAT box, but many of these recognise palindromic sequences and rely on surrounding nucleotides to bind. The NF-Y protein in contrast only requires these five nucleotides and it is the main protein that binds to the CCAAT box (Mantovani, 1999). The CCAAT box is one of the most prevalent elements that can be found in eukaryotic gene promoters and found in particular promoters involved in cell cycle regulation such as *cdc2*, CDC25C, Cyclin A and Cyclin B1 (Mantovani, 1998, Zwicker *et al.*, 1995a). Interestingly, these particular promoters are TATA-less and data show that there is an increased occurrence of CCAAT boxes in TATA-less promoters (Mantovani, 1998). These authors also show that the position of the CCAAT box is dependent on whether the promoter has a TATA box; the CCAAT box is situated nearer the transcriptional start site of the gene in the absence of a TATA box, whereas in the presence of a TATA box the

CCAAT box is situated on average between -80 and -100 bp upstream of the transcriptional start site. Notably the CCAAT box can also be found situated in promoters in the reverse orientation (Mantovani, 1998).

The NF-Y factor itself is made up of three subunits: NF-YA, NF-YB and NF-YC (Maity and de Crombrughe, 1998) and interacts with different transcription factors; for example TBP is found to have affinity for NF-YB and NF-YC (Bellorini *et al.*, 1997); Histone Acetyl-Transferases (HATs) are also associated with NF-Y subunits such as hGCN5 to the NF-YB-NF-YC complex (Currie, 1998) and P/CAF to NF-YA (Jin and Scotto, 1998) (for review see (Mantovani, 1999)). Both of these HATs act co-ordinately with NF-Y to activate transcription. NF-Y is also found to be essential in the binding of RNA Pol II to CCAAT box containing promoters and required for transcription by RNA Pol II (Kabe *et al.*, 2005).

NF-Y is a target for p53 and when in complex, these two proteins can negatively regulate G2/M promoters (Imbriano *et al.*, 2005). The fact that p53 binds to NF-Y is another mechanism by which p53 can mediate G2/M checkpoint arrest. Imbriano *et al.*, 2005 show that multiple CCAAT boxes are necessary for the repression of transcription induced by DNA damage. Furthermore, they show that p53 and NF-Y interact through the α -helix of NF-YC and the C-terminus of p53 (residues 355 to 375) the tetramerization domain of p53, sites acetylated by P/CAF and p300 respectively. p53 and NF-Y are co-resident on certain types of promoter, in particular those that are involved in the G2/M transition, which contain multiple CCAAT boxes and Imbriano *et al.*, 2005 note that after DNA damage the p53-NF-Y complex remains bound to the CCAAT box. Taken together these data suggest that p53 may induce transcriptional repression through the CCAAT box and that more

than one CCAAT box is required for repression. Acetylation status of both p53 and surrounding histones also plays a role in repression of transcription by p53 and NF-Y. The acetylation sites of p53 (K320, K373, and K382) become rapidly acetylated and HDACs are recruited to the complex and therefore surrounding histones are deacetylated. PCAF and p300, associated with both NF-Y and p53, are then released from the promoters. However, the CCAAT box bound p53-NF-Y complex is required but not sufficient for HDAC recruitment and repression (Imbriano *et al.*, 2005). Other factors that bind to NF-Y and inhibit transcription through the CCAAT box include the p53 family member, p63 (Testoni and Mantovani, 2006).

Through the interaction of p53 with NF-Y and the CCAAT box, p53 can repress promoters such as *cdc2* (Yun *et al.*, 1999), Cyclin B and *CDC25C* (Manni *et al.*, 2001) and therefore create a G2/M checkpoint arrest when DNA damage has occurred. Therefore NF-Y and the CCAAT box are important in p53 regulated transcription.

1.7.4 E2F binding site: p21 mediated negative regulation of E2F by p53

Another element through which p53 can mediate its regulation of gene expression is indirectly through the E2F binding site. The E2F binding site consists of the DNA sequence: TTTCGCGC (Zheng *et al.*, 1999). Depending on the stage of the cell cycle; which member of the E2F family binds to the consensus sequence; and the actual sequence itself, gene expression by E2F can either be activated or repressed (Araki *et al.*, 2003, Wells *et al.*, 2000). As the E2F binding site is similar to the CDE element it is proposed that the E2F binding site alongside CHR represses

transcription whilst E2F alone activates transcription (Zwicker and Muller, 1997). The effect different E2F family members have on transcription also has a bearing on whether gene transcription is activated or repressed. For example, the presence of E2F-4 (in complex with the Rb-related protein, p130) on the *cdc2* promoter in cells that are undergoing G1 phase represses expression of *cdc2* until the complex is released during S phase (Tommasi and Pfeifer, 1995). It is shown that the binding of Rb to E2F-1 inhibits the trans-activation of E2F-1 (Helin *et al.*, 1993a). Furthermore Rb also recruits histone deacetylase to repress transcription (Brehm *et al.*, 1998). E2F homo-dimerizes with other members of the E2F family or hetero-dimerizes with a close relative, DP1, which enhances the binding of E2F to the binding site and enhances trans-activation of gene expression (Helin *et al.*, 1993b). These authors also show that this complex is also inhibited by Rb.

E2F binding sites are included in many promoters most of which are mainly involved in cell cycle regulation and DNA replication, such as *cdc2* (Tommasi and Pfeifer, 1995), Cyclin A (Schulze *et al.*, 1995), Cyclin E (Botz *et al.*, 1996) and DNA Polymerase α (Pearson *et al.*, 1991). Therefore the involvement of E2F in these cellular mechanisms makes E2F a target for many proteins, such as p21 (expression of which is dependent upon p53), to inhibit cell cycle progression after stress or DNA damage.

p21, as discussed earlier, is involved in the control of E2F transcriptional activity through direct binding (Delavaine and La Thangue, 1999) or through the inhibition of CDKs (CDKs phosphorylate and inactivate Rb). p21 mediated activation of Rb allows Rb to bind to and inhibit E2Fs (Weinberg, 1995). p21 also disrupts the CDK2-p130-E2F1 complex allowing p130-E2F1 to repress transcription (Shiyanov

et al., 1996). Taken together these data and the fact that p21 expression is p53 dependent, E2F (and the E2F binding site) is, in effect, an indirect target of p53. Notably, the E2F binding partner DP is also inhibited by p53 (Gopalkrishnan *et al.*, 1998).

Together these data show that, through the E2F binding site, p53 can inhibit cell cycle progression and that this is another example by which p53 can induce cell cycle checkpoint arrest through other binding sites other than the p53 responsive element.

1.7.5 Sp1 Sites

The Sp1 transcription factor binds to sites that are GC rich or GT rich: the GC box, GGGGCGGGG (on occasion GGCGGG will suffice alone, for example in the PLK1 promoter (Uchiumi *et al.*, 1997)) and GT/CACCC box GGTGTGGGG (Suske, 1999). These sites are found throughout many promoters including PLK1, CDC25C (Zwicker *et al.*, 1995a) and p21 (Koutsodontis *et al.*, 2002). In the p21 promoter in particular, there are six proposed Sp1 sites that have the sequence similar to GGCGGG and are bound by specific members of the Sp1 family. Koutsodontis *et al.*, 2002, show that Sp1 and Sp3 are trans-activators of p21 expression and deletion of element 1, or a mutant of Sp1, reduced trans-activation. Also mutation of Sp1 binding element 3 almost abolished all p21 promoter activity. Sp1 trans-activation of p21 expression is enhanced by the cytokine TGF- β (Transforming Growth Factor, which inhibits cell cycle progression), mediated via the Smad signalling pathway. Mutation of Smad inhibits p21 induction by TGF- β (Moustakas and Kardassis, 1998,

Pardali *et al.*, 2000). These authors show that activation of p21 requires the binding sites for Sp1 on the promoter and that Smad and Sp1 cooperate at the GC rich Sp1 site in the p21 promoter to trans-activate p21.

p53 also co-operates acts with Sp1 to trans-activate the p21 promoter (Koutsodontis *et al.*, 2001). These authors show that 1) Sp1 acts independently of p53 to trans-activate the p21 promoter and 2) p53 stimulates p21 expression but requires Sp1 to do this, as mutation of either the p53 responsive element or the Sp1 site reduced p21 trans-activation. Further analysis showed that the p53 binding site is an enhancer of the proximal promoter which contains the Sp1 binding sites and that at the Sp1 binding site p53 interacts with Sp1 to trans-activate p21. Overall these data suggest that Sp1 and p53 act in co-operation with each other (or synergism) as a mechanism to regulate transcriptional activation of p53 target genes (Koutsodontis *et al.*, 2001).

Further to the above, the importance of the Sp1 site in promoters for transcriptional activation is highlighted by the fact that Sp1 interacts with a subunit of the TFIID complex, TAF(II)110 (Gill *et al.*, 1994) . Mutation of certain Sp1 binding sites reduced the binding of TAF(II)110 and trans-activation of gene expression (Gill *et al.*, 1994). Similarly, Sp1 interacts with E2F (Lin *et al.*, 1996). Both these proteins bind to one another and cooperate with each other to activate transcription. Data show that Sp1 increases transcription at a promoter containing only an E2F binding site and E2F increases transcription at a promoter containing only Sp1 sites (Lin *et al.*, 1996). Together these data show the interaction Sp1 has with E2F to activate transcription.

Many publications document the trans-activation capabilities of Sp1, however more recent data show that Sp1 (and other members of the Sp family, such as Sp3 and

Sp4) can repress transcription. Innocente *et al.*, 2005 show that p53 is able to bind to Sp1 and the Sp1 binding site to repress Cyclin B1 transcription and this is p21 and NF-Y (CCAAT box) independent. The Sp1 binding site is crucial in the repression of Cyclin B transcription as mutation of this abolishes repression (Innocente and Lee, 2005). Interestingly, growing evidence suggests that Sp1 is responsible for activation of gene transcription and that Sp3 and Sp4 compete with Sp1 for the Sp1 binding site and in doing so decrease the activation of transcription by Sp1 (Kwon *et al.*, 1999).

Again, it is shown that p53 can activate or indeed repress target genes through promoter elements and their component proteins and, in particular, p53 can do this in co-operation with Sp1 and the Sp1 binding site.

1.8 Polo-like Kinase-1 (PLK1)

This thesis focuses on p53 and the mechanism of its repression of proteins involved in the progression of G2 and M phase, specifically PLK1. The Sections below give an overview of PLK1, including the following: its function and cellular localisation, regulation throughout the cell cycle, down-regulation after DNA damage, PLK1 knockdown and depletion.

1.8.1 PLK Family

PLKs are a group of ser/thr kinases that cooperate with CDKs to regulate progression of the cell cycle (Strebhardt and Ullrich, 2006). First described in

Drosophila melanogaster, the mammalian family consist of four PLKs consisting of PLK1, PLK2 (Serum inducible kinase, Snk), PLK3 (proliferation-related kinase, Prk) and PLK4 (Snk akin kinase, Sak) (Barr *et al.*, 2004, Dai, 2005). PLKs are similar in structure to one another and contain two Polo-box domains (PDBs) and a ser/thr kinase domain. PLK4 however contains only one PBD. The function of PLK1 has been extensively studied and is involved in many cellular functions. This will be discussed in more detail in the next Section. The function of the other members of the family remains relatively elusive. Interestingly PLK2 levels and activity increases during the G1 phase of the cycle indicating that it is required for the progression of the cell cycle through G1 and S phase (Ma *et al.*, 2003). Silencing of PLK2 causes mitotic catastrophe and apoptosis (Burns *et al.*, 2003). Like that of PLK1, PLK3 and PLK4 levels increase in G2 and M phase of the cell cycle, however constitutive expression of PLK1 transforms NIH3T3 cells whereas constitutive expression of PLK3 inhibits proliferation by impeding cytokinesis (Conn *et al.*, 2000, Sillibourne and Bornens, 2010). Recent studies have demonstrated that PLK3 is activated during DNA damage checkpoint arrest and inhibits entry into mitosis through phosphorylation of CDC25C (Xie *et al.*, 2001). PLK3 transcription is activated by p53 through a p53 responsive element in the promoter region of the PLK3 gene (Jen and Cheung, 2005). Notably PLK2 is also a target of p53 transcriptional activity and the promoter region of the PLK2 gene contains three p53 responsive elements. Data show that p53 activates PLK2 expression through two of these sites and represses transcription through the third element (Burns *et al.*, 2003). PLK4 is also repressed by p53 (Li *et al.*, 2005).

The data currently available for the PLK family demonstrate that PLK2 and PLK3 are transcriptional targets of p53. PLK1 is the most studied of all the members of the

PLK family and is required for many cellular processes needed for cell cycle progression and although it has been shown to be down-regulated in a DNA damage dependent manner (Smits *et al.*, 2000) it is not known whether p53 directly represses PLK1 transcription. The next Sections aim to discuss the current knowledge surrounding PLK1 function and its down-regulation by DNA damage.

1.8.2 Function and localisation of PLK1

PLK1 is a protein kinase that phosphorylates many substrates, some of which are summarised in Figure 1.6, and is localised to many cellular structures which are also shown in Figure 1.6.

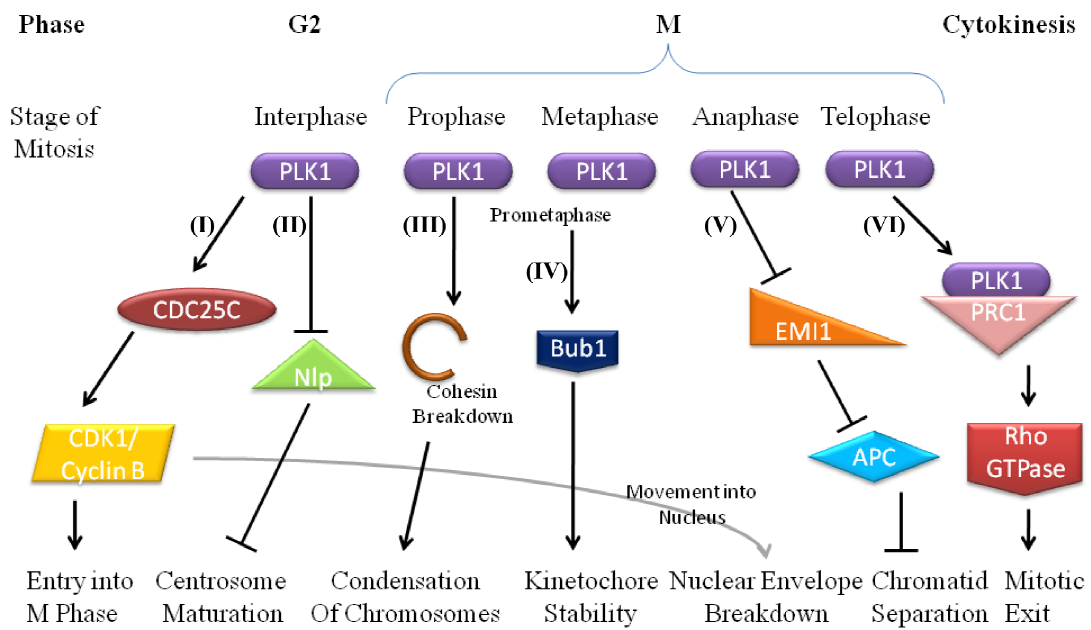


Figure 1.6: Function and localisation of PLK1

PLK1 is involved in various functions in the mitosis phase of the cell cycle. Figure derived from various authors (Archambault and Glover, 2009, Pines, 2002, Strebhardt and Ullrich, 2006). (I) PLK1 phosphorylates CDC25C which in turn dephosphorylates and activates the CDK1/Cyclin B complex (II) PLK1 phosphorylates Nlp, which dissociates from the centrosome so allow centrosome to mature (III) PLK1 mediates cohesion breakdown (IV) PLK1 phosphorylates Bub1 which is needed for kinetochores stability (V) PLK1 stimulates the degradation of the Early Mitotic Inhibitor 1 (EMI1), an anaphase promoting complex (APC) inhibitor and (VI) PLK1 binds to the central spindle by an interaction with Protein Regulator of Cytokinesis 1 (PRC1). PLK1 activates a Rho GTPase that stimulates the contraction of the contractile ring required for cytokinesis.

PLK1 was first described by the finding that mutant alleles of Polo (PLK1) caused significant aberration of mitotic spindles and abnormal segregation of chromosomes in *Drosophila* (Sunkel and Glover, 1988). Mutants of the Polo homologue in *S.pombe*, plo, cannot mediate chromosome segregation and the PLK1 homologue in *S.cerevisiae*, cdc5, mutation also leads to a mitotic arrest (Ohi *et al.*, 1994, Ohkura *et al.*, 1995). The name Polo was derived from the fact that the bipolar spindle (that aligns the chromosomes in pairs in the centre of the mitotic spindle), is impaired in the absence of PLK1, with the outcome that chromosomes simply align around the mono-polar spindle, which encircles the mitotic spindle (Sunkel and Glover, 1988).

PLK1 has many roles to play in the latter stages of G2 phase, mitotic entry and throughout mitosis and even cytokinesis (Petronczki *et al.*, 2008). It is localised to various cellular structures including the centrosomes in prophase, kinetochores in prometaphase and metaphase, central spindle in anaphase, and it also accumulates in the midbody during telophase (Petronczki *et al.*, 2008). It is thought that PLK1 recognises substrates through its PBDs, which recognise particular phospho-peptide sequences on the substrate (i.e. the PBDs, Polo Box Domains). Mutation of either of the PBDs causes catastrophic mitotic events, such as randomly orientated spindles in prophase and in metaphase and randomly orientated bipolar spindles (Seong *et al.*, 2002). Overall the mutation caused destabilisation of mitotic spindles and cytokinesis failure (Seong *et al.*, 2002). Furthermore, mutation inhibited PLK1 association with the spindle poles and cytokinetic neck filaments (Lee *et al.*, 1998). Interestingly, it was shown that deletion of the C-terminus (now known to harbour the PBD) increased the kinase activity of PLK1 significantly, therefore showing that the PBD has a role in the regulation of PLK1 activity (Mundt *et al.*, 1997).

PLK1 is a well known protein kinase and is responsible for phosphorylation of diverse number substrate proteins, such as CDC25C and Cyclin B1. The exact mechanism by which PLK1 targets its substrates and how the PBD might facilitate this is not clearly understood. Some authors suggest that the PBD domain inhibits the kinase domain until a phospho-peptide binds to the PBD and which releases the kinase domain (Lowery *et al.*, 2005). They also suggest that there are two models by which PLK1 targets its substrates: either 1) PLK1 PBD recognises the phospho-peptide of a scaffold protein, located at a cellular structure, allowing the kinase domain to phosphorylate neighbouring substrates located at the cellular structure 2) the PLK1 PBD binds to a substrate phospho-peptide, which then allows the kinase

domain to further phosphorylate and activate that substrate (Lowery *et al.*, 2005, Lowery *et al.*, 2004). The difference in the models is that the PDB either recognises the substrate and directly binds to the substrate to phosphorylate the substrate, or recognises a scaffold protein that brings PLK1 to a particular structure in the cell and phosphorylates the substrate without actually binding to it.

PLK1 plays a large role at the centrosomes of the cell. For example, it is responsible for the phosphorylation of the centrosomal protein Nlp (Ninein-like protein, which regulates microtubule nucleation). The Nlp protein function is to manage microtubule minus-end anchoring and nucleation events during interphase, and phosphorylation by PLK1 dissociates Nlp from the centrosome in mitosis. This is to presumably inhibit the formation of new microtubules and stabilises the already formed microtubules (Casenghi *et al.*, 2005, Casenghi *et al.*, 2003). Another substrate of PLK1 that is important for entry into mitosis is CDC25C. PLK1 binds to phosphorylated CDC25C (which is primed by a phosphorylation group by CDK1/Cyclin B complex) through its PBD domain and phosphorylates CDC25C to activate CDC25C. Interestingly, mutation of the PBD inhibits the binding of PLK1 to CDC25C and inhibits centrosomal localisation of PLK1 (Elia *et al.*, 2003a, Elia *et al.*, 2003b).

Another cellular structure at which PLK1 is important is at the kinetochore (the protein structure on chromosomes where microtubules attach during division and is responsible for the separation of chromosomes). PLK1 is required for the attachment of the microtubules to the kinetochores and this can be inhibited by the PLK1 inhibitor, BI 2536 (Elowe *et al.*, 2007). A particular PLK1 substrate involved at the kinetochore and microtubule interface is Bub1. PLK1 binds to pre-phosphorylated

Bub1 (primed by a phospho group by CDK1), and further phosphorylates Bub1 at residue S676 creating kinetochore stability (Elowe *et al.*, 2007). These authors also show that phosphorylation of Bub1 by PLK1 occurs at prometaphase and is removed in metaphase.

Taken together, these data show that PLK1 has many important roles to play in the cell cycle, particularly in mitosis and is associated with many mitotic structures, only some of which have been touched upon above. They also show that PLK1 is vital in the correct management of mitosis and without it, the integrity of the genome is lost and therefore cell cycle progression cannot proceed.

1.8.3 Cell cycle regulation of PLK1

The regulation of PLK1 is well documented and was first observed by two groups who show that the activity and levels of PLK1 increase at the end of G2 phase and reach a maximum level in mitosis (Golsteyn *et al.*, 1995, Hamanaka *et al.*, 1995). At the end of mitosis PLK1 activity and levels decrease. The factor that is responsible for cell cycle regulation of PLK1 levels was identified as being the CDE/CHR element in the promoter of PLK1 (Uchiumi *et al.*, 1997), which was previously identified in the cell cycle regulated, G2 and M phase protein, CDC25C (Lucibello *et al.*, 1995, Zwicker *et al.*, 1995b). Uchiumi *et al.*, 1997 show comprehensively the elements in the PLK1 promoter that are responsible for the activation and repression of PLK1 expression. They show that activation of PLK1 expression is due to a Sp1 site, CCAAT box and an element neighbouring and upstream of the Sp1 binding site. Mutation of the Sp1 binding site reduces promoter-driven Luciferase expression and

removal of the site reduces this further. Mutation or removal of the CCAAT box almost abolishes all Luciferase activity (Uchiumi *et al.*, 1997). Furthermore, these authors show that cell cycle regulation is regulated by a CDE/CHR element and mutation of the CDE or CHR element increases promoter-driven Luciferase expression during G1 phase indicating that both elements are involved in PLK1 transcriptional repression during this phase under normal conditions. Interestingly, the CHR mutant alone had a much larger level of promoter-driven Luciferase expression in G1 phase extracts compared to the CDE mutant alone. These data demonstrate that CHR has the larger role to play in repression of PLK1 during the G1 phase of the cell cycle, compared to the CDE.

Later publications also show the importance of the E2F binding site in PLK1 (130 base pairs upstream from the transcriptional start site) and regulation by other proteins such as RB and the SWI/SNF (chromatin remodelling) complex. As E2F members can either activate or repress transcription, the role of the E2F binding site of PLK1 was unclear, although it was assumed it was more likely to play a repressive role at the PLK1 promoter, like that of the G2/M promoter of *cdc2* (Tommasi and Pfeifer, 1995). E2F binding site deletion does not affect activation of transcription of PLK1 (Zhu *et al.*, 2002) and data show that the repressive member of the E2F family, E2F4, binds to the E2F binding site in-conjunction with the Rb proteins, p107 and p130 and together represses transcription (Gunawardena *et al.*, 2004). In the presence of Rb, the SWI/SNF histone deacetylation complex is recruited and alongside of this complex E2F is able to repress the PLK1 promoter further. Notably, the E2F-Rb complex alone could not repress PLK1 transcription to the same extent as in the presence of SWI/SNF (Gunawardena *et al.*, 2004) (for reviews see (Archambault and Glover, 2009, Martin and Strebhardt, 2006)). The

mechanism by which Rb recruits SWI/SNF to repress transcription also occurs at the promoters of Cyclin E and *cdc2* (Siddiqui *et al.*, 2003, Zhang *et al.*, 2000). Although the importance of Rb alone in repression of PLK1 is further illustrated by the fact that repression is abrogated in the absence of all three Rb members (Rb, 107 and 130) (Jackson *et al.*, 2005).

There are many mechanisms by which PLK1 transcription is regulated, however the purpose of this thesis is to ask the question of how is PLK1 down-regulated and is down-regulation p53 dependent? The next Section aims to discuss the recent data showing that PLK1 is down-regulated after DNA damage.

1.8.4 PLK1 down-regulation by DNA damage and PLK1 Depletion/Knockdown

Studies show that DNA damage induces the down-regulation of PLK1. In particular, DNA damage induced by Adriamycin and Cisplatin, was shown to down-regulate PLK1 and that this caused a block of the cell cycle at G2 and in Mitosis (Ando *et al.*, 2004, Smits *et al.*, 2000). DNA damage also down-regulates PLK1 mRNA expression suggesting that PLK1 is repressed at the gene level (Ree *et al.*, 2003). Although the mechanism by which PLK1 is repressed by DNA damage is unclear these authors suggest that there is an involvement of BRCA1 in PLK1 repression. PLK1 down-regulation is also ATM and ATR dependent (van Vugt *et al.*, 2001) and occurs concomitantly with increases in p53 and p21 suggesting an involvement of p53 in PLK1 down-regulation (Incassati *et al.*, 2006, Kho *et al.*, 2004, Sur *et al.*, 2009). Kho *et al.*, 2004 show that p53 repression of PLK1 occurs in a p53 wild type

cell line and cannot in the p53-null equivalent cell line, when cells are treated with 5-Flurouracil.

Not only are protein and mRNA levels down-regulated by DNA damage, PLK1 activity is inhibited by DNA damage (Smits *et al.*, 2000). This indicates that DNA damage inhibits PLK1 activity as well as down-regulating or repressing PLK1.

Interestingly, expression of a mutant form of PLK1 that is constitutively active over-rides the DNA damage induced G2 arrest (Smits *et al.*, 2000). Data demonstrate that PLK1 is essential for the progression of mitosis in DNA damaged cells, unlike in undamaged cells, and required for the degradation of Wee1 (inactivates CDK1), which itself is needed for cell cycle progression (van Vugt and Medema, 2004, van Vugt *et al.*, 2004).

PLK1 repression is a target of DNA damage. PLK1 knockdown is shown to cause considerable inhibition of cellular growth at G2 and M, inhibition of centrosome maturation, inhibition of spindle formation and an increase in the number of cells in apoptosis (Liu and Erikson, 2002, Spankuch-Schmitt *et al.*, 2002a, Spankuch-Schmitt *et al.*, 2002b). Interestingly, data show that PLK1 depletion induces increased mitotic arrest, and reduced cell survival in the p53-null cell lines suggesting that p53 plays a protective role under PLK1 depletion conditions (Guan *et al.*, 2005). p53-null cells are sensitive to PLK1 depletion and undergo an increased cell death (Liu *et al.*, 2006). Furthermore, PLK1 depletion is reported to promote DNA damage (Liu and Erikson, 2003, Yim and Erikson, 2009).

Taken together these data illustrate that DNA damage induces PLK1 repression and that this may be due to p53. However, evidence shows that the mechanism by which

p53 does this cannot be replicated by depleting PLK1 and that p53 protects the cell under these conditions. The mechanism by which p53 represses PLK1 is unclear and this thesis aims to understand how p53 may do this.

1.8.5 PLK1 and cancer

PLK1 is found to be overexpressed in many cancers including breast, ovarian, colorectal and non-Hodgkin lymphoma's (for review see (Strebhardt and Ullrich, 2006)). Furthermore, transfection and overexpression of PLK1 into NIH3T3 cells causes malignant transformation of these cells (Smith *et al.*, 1997) and therefore is a very important potential therapeutic target.

What makes this target even more interesting is the fact that in cells expressing mutant p53 (p53 mutation that occurs in around 50% of human cancers), PLK1 inhibition/knockdown leads to reduction in the survival of these cells and increased mitotic arrest in these cells, compared to their wild type counterparts (Guan *et al.*, 2005). Other authors also show that in p53 defective cell lines that have been treated with Ionising Radiation, PLK1 levels increase suggesting that p53 regulates PLK1 in stressed conditions (Sur *et al.*, 2009). They also note that PLK1 inhibitors are much more effective in p53 deficient cells.

Taken together the data above suggest that targeting PLK1 in p53 deficient tumours has significant potential as a cancer therapy. However it has also been shown that PLK1 depletion can cause DNA damage (Liu and Erikson, 2003, Yim and Erikson, 2009) and tumour formation (Lu and Yu, 2009) suggesting that, in fact, a normal PLK1 level must need to be maintained in tumour cells.

1.9 Thesis Aims

The model shown in Figure 1.7 is a representation of the mechanism(s) we believe that p53 utilises to down-regulate PLK1 at the G1/S checkpoint and most importantly the G2/M checkpoint.

This thesis aims to decipher between two possible mechanisms by which p53 down-regulates PLK1. The first is through CDK inhibition by the p53 inducible CDK inhibitor, p21. p21 arrests the cell cycle at the G1/S checkpoint. In some circumstances, repression by p21 is also mediated through the CDE/CHR element therefore this thesis asks how key the role of p21 has to play in PLK1 down-regulation or down-regulation through the G1/S checkpoint? Secondly, this thesis aims to seek out whether there is a mechanism involving direct p53 binding to the PLK1 promoter which represses PLK1. There is supporting evidence for direct repression by p53. Here I explore this mechanistically and identify two possible REs in the promoter. Therefore does p53 bind to one or both of these? Also what is the role of the CDE/CHR element at this stage and can p53 or p21 repress PLK1 expression sufficiently through this element? Overall, we wish to understand the extent of the dependency PLK1 repression has on p53 and is some of this repression down to p21?

PLK1 Repression: the model

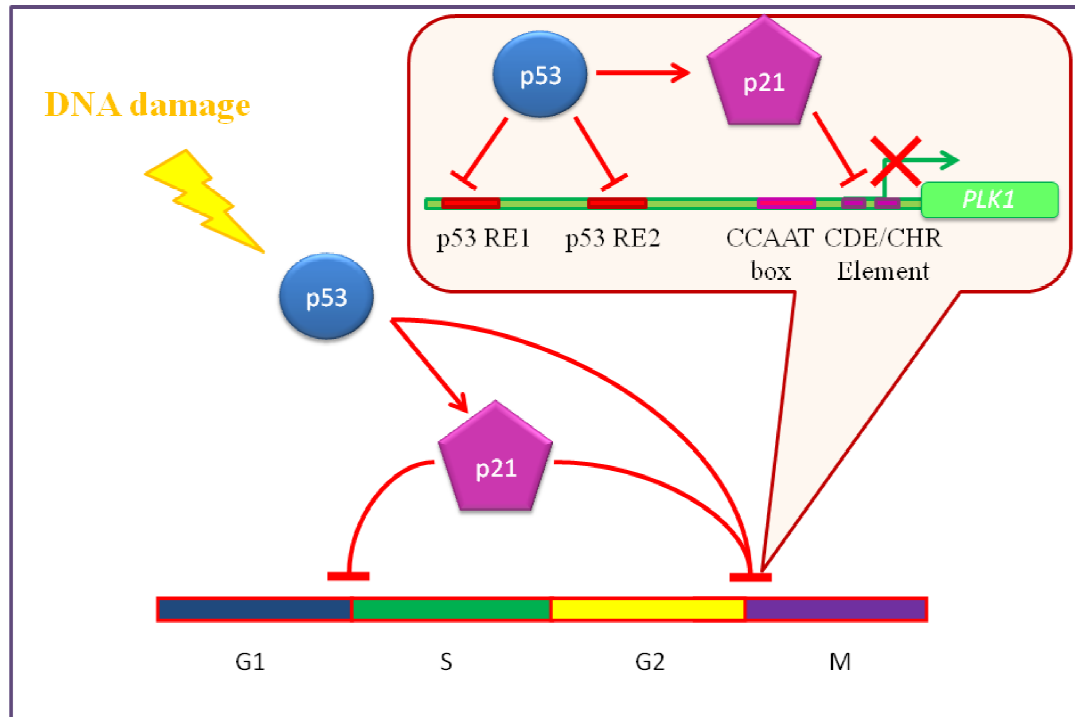


Figure 1.7: Proposed mechanisms of down-regulation and/or repression of PLK1 by p53 and p21

This Figure shows the proposed mechanism(s) by which p53 may down-regulate or repress PLK1. p53 mediates G1/S checkpoint arrest by inducing expression of p21 and therefore PLK1 down-regulation may be due to the fact that PLK1 levels are low in the G1 and S phases of the cell cycle. On the other hand, p53 may act to repress PLK1 by directly repressing expression of PLK1 through proposed p53 responsive elements at the PLK1 promoter. p21 transcriptional activity may also repress PLK1 expression through the CDE/CHR element.

Chapter 2: Materials and Methods

2.1 Reagents and Buffers

General chemicals were purchased from Sigma Aldrich unless otherwise stated.

Deionised water (MilliQ) was used throughout.

Agarose Gel Loading Buffer (5X)

40 % (w/v) Sucrose

0.25 % (w/v) Bromophenol Blue

Buffer P1 (Sterile)

50 mM Tris pH 8

10 mM EDTA

100 µg/ml RNase A

Buffer P2 (Sterile)

0.2 M NaOH

1 % SDS

Buffer P3 (Sterile)

3 M KOAc pH 5.5

ChIP Collection Buffer

1 X PBS

1 X Protease Inhibitor Cocktail 1 (Calbiochem)

ChIP SDS Lysis Buffer

50 mM Tris-HCl pH 8

10 mM EDTA pH 8

1 % (w/v) SDS

1 X Complete, EDTA-free Protease Inhibitor Cocktail (Roche)

ChIP Dilution Buffer

20 mM Tris pH 8

150 mM NaCl

2 mM EDTA pH 8

1 % (v/v) Triton X-100

1 X Complete, EDTA-free Protease Inhibitor Cocktail (Roche)

ChIP Low Salt Buffer

20 mM Tris pH 8

150 mM NaCl

2 mM EDTA pH 8

1 % (v/v) Triton X-100

0.1 % (w/v) SDS

1 X Complete, EDTA-free Protease Inhibitor Cocktail (Roche)

ChIP High Salt Buffer

20 mM Tris pH 8

500 mM NaCl

2 mM EDTA pH 8

1 % (v/v) Triton X-100

0.1 % (w/v) SDS

1 X Complete, EDTA-free Protease Inhibitor Cocktail (Roche)

LiCl Buffer

250 mM LiCl

10 mM Tris pH 8

1 mM EDTA pH 8

1 % (v/v) Igepal

1 % (w/v) Na Deoxycholate

EMSA p53 Binding Buffer

50 % (v/v) Glycerol

250 mM KCl

100 mM HEPES pH 7.4

5 mM DTT

5 mg/ml BSA

0.5 % (v/v) Triton X-100

Enhanced Chemiluminescence (ECL) Solution 1

2.5 mM Luminol

396 μ M p-coumaric acid

100 mM Tris, pH 8.5

Enhanced Chemiluminescence (ECL) Solution 2

0.0192 % (v/v) H₂O₂

100 mM Tris, pH 8.5

Flow Cytometry Antibody Buffer

1 X PBS

0.5 % (w/v) BSA

Non-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (non-SDS-PAGE) Running Buffer (10 X)

250 mM Tris

1.92 M Glycine

SOB Broth

2 % (w/v) Tryptone

0.5 % (w/v) Yeast Extract

0.05 % (w/v) NaCl

10 mM MgCl₂

10 mM MgSO₄

SOC Medium

0.02 mM Glucose

SOB Broth

SDS Sample Buffer (5 X)

125 mM Tris-HCl pH 6.8

4 % (w/v) SDS

20 % (v/v) Glycerol

0.2 M DTT

0.1 % (w/v) Bromophenol Blue

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**Running Buffer (10 X)**

250 mM Tris

1.92 M Glycine

1 % (w/v) SDS

TE (1X)

10 mM Tris pH 8

1 mM EDTA pH 8

Transformation Buffer 1, pH 5.8

100 mM RbCl

50 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$

30 mM $\text{KC}_2\text{H}_3\text{O}_2$, pH 7.5

10 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

15 % (v/v) glycerol

Transformation Buffer 2

10 mM 3-(N-morpholino) propanesulphonic acid (MOPS), pH 6.8

10 mM RbCl

74 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

15 % (v/v) glycerol

Tris-acetate EDTA (TAE) Buffer (50 X)

2 M Tris-base

5.7 % (v/v) Glacial Acetic Acid

0.1 M Na₂EDTA.2H₂O, pH 8.0

Tris-borate EDTA (TBE) Buffer (10X)

0.45 M Tris-base

0.44 M Orthoboric acid

10 mM Na₂EDTA pH 8.0

Trypsin-EDTA (Sterile)

Trypsin 0.25 % (Gibco)

1 X PBS

1 mM EDTA pH 8.0

Western Blocking Buffer

5 % (w/v) Marvel dried milk

0.1 % (v/v) Tween 20

1 X PBS

(Light) Western Blocking Buffer

3 % (w/v) Marvel dried milk

0.5 % (v/v) Tween 20

1 X PBS

Western Semi-dry Transfer Buffer

25 mM Tris base

192 mM Glycine

0.1 % (w/v) SDS

20 % (v/v) Methanol

Western Wet Transfer Buffer

25 mM Tris base

192 mM Glycine

20 % (v/v) Methanol

Western Washing Buffer

1 X PBS

0.1 % (v/v) Tween 20

2.2 Cell Culture and Related Techniques

2.2.1 Cell culture maintenance

Cells lines, described in Table 2.1, were cultured in a humidified 5 % CO₂ atmosphere at 37 °C. Stocks of H1299, SAOS2 and U2OS cells, all available cells lines in the laboratory, were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco). DMEM was supplemented with 10 % (v/v) fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (all Gibco). HCT116 p53 wild type, p53-null and p21-null were cultured in McCoy's 5A medium (Biosera) which was supplemented with 10 % (v/v) fetal bovine serum (FBS, Biosera) and 2 mM L-glutamine. When cells reached 90 % confluency they were sub-cultured, as detailed in Table 2.1. Adherent cells were washed twice in PBS (37 °C) and then detached from the plates using Trypsin-EDTA (see Section 2.1 Reagents and Buffers). Detached cells were re-suspended in medium and transferred to new plates/flasks with the appropriate fresh medium.

Cell Line	Derivation	Subculture	ATCC® Number
H1299	Human non-small cell lung carcinoma - p53 null	1:8	CRL-5803
HCT116 (wild type)	Human colon carcinoma	1:6	CCL-247
SAOS2	Human osteogenic sarcoma – Rb and p53 null	1:2	HTB-85
U2OS	Human osteosarcoma - ARF null	1:4	HTB-96
HCT116 (p53-null, p21-null)	Human colon carcinoma	1:6	Vogelstein Laboratory*

Table 2.1: Mammalian cell lines

*Dr. Vogelstein, The Johns Hopkins University, USA

2.2.2 Cell Seeding

Cell lines were seeded by washing and trypsinising the cells. They were then pooled together into a suspension, 10 µl of which was added to each end of a haemocytometer (Hawksley, 1/400m²). The cells were counted under the microscope and seeded as required.

2.2.3 Drug Time Course

Cells (HCT116 together with p53-null and p21-null derivatives, B.Vogelstein) were seeded at a density of 1×10^6 per 10 cm plate. After 24 h, Etoposide (final concentration 100 µM), Nutlin-3 (final concentration 10 µM) or Cisplatin (final

Concentration 20 μM), all solubilised in DMSO (Dimethyl Sulfoxide), were added directly to the cell medium. Cells were then lysed in 500 μl of 5 X Sample Buffer at 2 h intervals over a 10 h period, with the final time point at 24 h. As controls, cells were untreated or treated with 0.1 % DMSO (Dimethyl Sulfoxide) and lysed in 500 μl of 5 X Sample Buffer. This was to account for any effects the DMSO alone is having on the cells. The samples were then analysed by SDS-PAGE and Western blotting.

2.2.4 Drug Titre

Cells (HCT116, together with p53-null and p21-null derivatives, B.Vogelstein) were seeded at a density of 1×10^6 per 10 cm plate. After 24 h, Etoposide was added to individual plates to give final concentrations of 5 μM , 10 μM , 20 μM , 50 μM and 100 μM . After 24 h each plate of cells was lysed in 500 μl of 5 X sample buffer. This method was also carried out with Nutlin-3 at 1 μM , 2 μM , 5 μM and 10 μM (final concentration) and with Cisplatin at 20 μM , 50 μM , 100 μM and 120 μM (final concentration). A plate of untreated cells was also lysed as well as a plate of cells treated for 24 h with 0.1 % DMSO, to account for any effects the DMSO is having on the cells. The samples were then analysed by SDS-PAGE and Western blotting.

2.2.5 siRNA Transfection

p21 siRNA tranfection was performed in both HCT116 wild type cells and U2OS cells. *PLK1* siRNA was performed in HCT116 wild type cells and their p53-null and

p21-null derivatives. siRNA was transfected into HCT116 cells and the p53-null and p21-null derivatives, using Lipofectamine RNAiMAX (Invitrogen) transfection reagent, as instructed by the manufacturer. U2OS were transfected using Lipofectamine 2000 (Invitrogen) transfection reagent as described by the manufacturer. The siRNA (Thermo Scientific) used is described in Table 2.2. The siRNA comprises a pool containing four siRNA oligos.

HCT116 wild type cells and the p53-null and p21-null derivatives were seeded at a density of 1×10^6 per 10 cm plate. After 24 h, 12 μ l of a 20 μ M stock of siRNA was added to 4 ml Opti-MEM® I Reduced Serum Media (Gibco). 40 μ l Lipofectamine RNAiMAX was then added and the mixture incubated at room temperature for 20 min. The medium was removed from the cells and replaced with the transfection mixture together with 10 ml fresh media (siRNA final concentration – 30 pmol).

U2OS cells were seeded at a density of 1×10^6 per 10 cm plate. After 24 h, 40 μ l Lipofectamine 2000 was added to 800 μ l Opti-MEM (mix 1) and incubated at room temperature for 5 min. 40 μ l siRNA (20 μ M) was as added to 800 μ l Opti-MEM (mix 2). Mix 1 was added to mix 2 and incubated at room temperature for 15 min. Media on the cells was removed and replaced with the transfection mixture added to up to 10 ml with fresh media (siRNA final concentration – 100 pmol).

Post transfection cells were incubated for 48 h before lysing in 500 μ l 5 X sample buffer or analysed by flow cytometry, as described in Section 2.2.10.

Name	Sequence	Target
ON-TARGETplus SMARTpool siRNA J-003290-09	GCACAUACCGCCUGAGUCU	human PLK1
ON-TARGETplus SMARTpool siRNA J-003290-010	CCACCAAGGUUUUCGAUUG	human PLK1
ON-TARGETplus SMARTpool siRNA J-003290-11	GCUCUUCAAUGACUCAACA	human PLK1
ON-TARGETplus SMARTpool siRNA J-003290-12	UCUCAAGGCCUCCUAAUAG	human PLK1
ON-TARGETplus SMARTpool siRNA J-003471-09	CGACUGUGAUGCGCUAAUG	human CDKN1 (p21 CIP)
ON-TARGETplus SMARTpool siRNA J-003471-10	CCUAAUCCGCCCACAGGAA	human CDKN1 (p21 CIP)
ON-TARGETplus SMARTpool siRNA J-003471-11	CGUCAGAACCCAUGCGGCA	human CDKN1 (p21 CIP)
ON-TARGETplus SMARTpool siRNA J-003471-12	AGACCAGCAUGACAGAUUU	human CDKN1 (p21 CIP)

Table 2.2: ON-TARGETplus SMARTpool siRNA (ThermoScientific)

2.2.6 Double Thymidine Block and Release

HCT116 wild type cells were seeded at 2.8×10^5 per 10cm plate. After 24 h Thymidine was added to a final concentration of 2 mM for 16 h to block the cells at the G1/S phase of the cell cycle. To release cells from the cell cycle block, cells were washed twice with warm 1 X PBS and fresh medium was added for a further 8 h. A second block was again initiated by the addition of 2 mM Thymidine for 16 h and then cells released from the block by washing the cells with warm 1 X PBS and adding fresh medium. Cells were subsequently lysed in sample buffer (500 μ l) at 2 h intervals over a 24 h period and analysed by SDS-PAGE and Western blotting.

2.2.7 Histone Deacetylase Inhibition

Cells (HCT116 wild type, p53 null) were seeded at a density of 1×10^6 per 10 cm plate. After 24 h, Nutlin-3 was added to final concentration of 10 μ M and/or Trichostatin A was added to a final concentration of 1 μ M. Treatments were maintained for 16 h and cells lysed in 500 μ l of 5 X sample. As controls, cells were untreated or treated with DMSO for 16 h to account for any effects the DMSO (the vehicle for the drugs) is having on the cells, and then lysed in 500 μ l of 5 X sample buffer. The samples were then analysed by SDS-PAGE and Western blotting.

2.2.8 Luciferase Transfections

Cells (HCT116 and their derivatives p53-null and p21-null derivatives, SAOS2) were seeded at a density of 0.3×10^5 per well of a 24 well plate and incubated overnight at 37 °C. Cells were then transfected with plasmid DNA, as described in Table 2.3.

Luciferase transfections were performed using FuGENE® 6 Transfection Reagent (Roche) and each transfection performed in triplicate. 1 µg total DNA was used in each transfection (one transfection per well of cells). Total DNA was added to 50 µl serum free media (mix 1). Each µg of DNA required 3 µl FuGENE, therefore 3 µl of FuGENE was added to 47 µl serum free media and incubated at room temperature for 5 min (mix 2). This was done for each transfection. Mix 2 was added to mix 1 and left at room temperature for 20 min. Each transfection mixture was then added to a seeded well of cells and incubated overnight at 37 °C.

Cells were then lysed 24 h after transfection with 100 µl 1 X passive lysis buffer (Dual Luciferase Reporter Assay System, Promega).

Transfection	1	2	3	4	5	6	7
p53 plasmid (ng)	0	1	3	10	30	100	300
<i>PLK1</i>promoter/ pGL3 reporter plasmid (ng)	250	250	250	250	250	250	250
pCDNA3 empty expression plasmid (ng)	745	744	742	735	715	645	445
<i>SV-Renilla</i> plasmid (ng)	5	5	5	5	5	5	5

Table 2.3: Plasmid concentrations used in Luciferase tranfections of cells (each tranfection was performed in triplicate)

2.2.9 Luciferase Assay

The Luciferase assay was carried out by adding 20 µl of each sample into individual wells of a 96 well plate. The plate was then read by a Microumat LB 96V luminometer (Berthold EG&G Instrument), using Luciferase Assay Buffer/Substrate and Stop & Glo® Buffer/Substrate from the Dual Luciferase Reporter Assay System (Promega). Variations in transfection efficiencies were corrected by determining the ratio of activity of Luciferase to *Renilla* in the sample. The average of each triplicate of ratios was then taken and plotted in a graph. Standard deviation was calculated and added as error bars to the graph.

2.2.10 Flow Cytometry

In order to measure progression through the cell cycle cells (either having been treated with a drug or siRNA, etc), cells are stained with 5-bromo-2-deoxyuridine (BrdU) and Propidium Iodide (Invitrogen). BrdU is a synthetic nucleoside that is an analogue of Thymidine and incorporates into the DNA only when the cell is synthesizing new DNA in Synthesis Phase (S Phase), therefore it is a marker of cells in S Phase of the cell cycle. BrdU itself cannot be detected in flow cytometry analysis on its own and so flow cytometry requires the use of an anti-BrdU antibody (Becton Dickinson) and secondary anti-mouse-IgG-FITC-conjugated antibody.

Propidium Iodide is a fluorescent molecule that intercalates into DNA and is used to determine DNA content of the cell. By knowing the DNA content of a cell, the phase of the cell cycle a cell is in can also be determined. For example, cells in G2 Phase of the cell cycle have double the DNA content of those in G1 Phase of the cell cycle,

therefore there is double the amount of Propidium Iodide in a G2 Phase cell than that of a G1 Phase cell. This can be plotted and shown in a graph. When used together, these two reagents are used to define the progression of cells through the cell cycle.

Cells were labelled by adding BrdU to a final concentration of 30 μ M and incubated for 15 min at 37 °C. Cells were then washed with 1 X PBS and trypsinised, then centrifuged at 1000 rpm for 5 min. After centrifugation, cells were re-suspended in 1 ml of 1 X PBS to which 3 ml cold 100 % ethanol was added. Subsequently this was then incubated overnight at 4 °C. The suspension was centrifuged at 2500 rpm for 5 min and the pellet re-suspended in 2 ml pre-warmed pepsin (1 mg/ml) in 30 mM HCl (pH 1.5) and incubated at 37 °C for 30 min with agitation. Following another centrifugation step, the pellet was re-suspended in 1 ml of 2 M HCl and incubated at room temperature for 15 min, after which a further 1 ml of 1 X PBS was added and sample centrifuged. The pellet was once again washed with 1 X PBS, centrifuged and washed with flow cytometry antibody buffer (See Section 2.1 Reagents and Buffers). The pellet was re-suspended in 1 ml of antibody buffer containing Anti-BrdU antibody at a dilution of 1 in 50 and incubated at room temperature for 1 h. The volume in the tube was consequently made up to 1 ml 1 X PBS and centrifuged. The pellet was re-suspended in 1 ml antibody buffer containing anti-mouse IgG FITC conjugated antibody (diluted 1 in 64) and incubated at room temperature for 30 min. The volume in the tube was made up to 1 ml 1 X PBS and centrifuged, and then the pellet re-suspended in 500 μ l 1 X PBS containing 25 μ g/ml propidium iodide. The tubes were wrapped in aluminium foil and analysed on the fluorescence-activated cell sorter (FACScan flow cytometer, Becton Dickinson).

2.2.11 RNA Extraction

RNA extraction was carried out using the RNeasy Mini Kit and QIAshredder (Qiagen). RLT Lysis Buffer, supplied with the kit, was supplemented with 2-mercaptoethanol (4 % w/v final concentration). From this 250 µl was added to each plate of HCT116 (HCT116 and the p53-null and p21-null derivatives) cells. Each lysate was collected and put into individual QIAshredder mini spin columns and centrifuged at 14000 rpm for 2 min. 350 µl 70 % ethanol was added to the supernatant of each sample and each lysate put into individual RNeasy mini spin columns and centrifuged at 14000 rpm for 15 sec. The columns were washed with 700 µl RW1 Wash Buffer, the supernatants were discarded and then the columns were washed twice with 500 µl RPE Wash Buffer, discarding the supernatants after each centrifugation. The RNA was then eluted from each column with 30 µl RNase free ddH₂O.

The concentration of RNA was determined by measuring of the absorbance at 260 nm (A_{260}) in an Ultraspec 2000 UV/visible spectrophotometer (Pharmacia Biotech).

2.2.12 cDNA Synthesis

Total RNA extracted, as described in the previous Section, was used as the template for the production of cDNA. To ensure there was no DNA in the RNA sample a DNase reaction was carried out by incubating 1 µg RNA with 2 µl 5 X First Strand Buffer (Promega), 1 µl RQ1 DNase (Promega) made up to 10 µl with Diethylpyrocarbonate (DEPC) treated H₂O (RNase free water) (Fisher Scientific) and

subsequently incubated at 37 °C for 30 min. 1 µl of RQ1 DNase Stop Solution (Promega) was added to each sample which was then incubated at 65 °C for 10 min to stop the reaction.

cDNA was synthesised by reverse transcription, this reaction was initiated by adding 11 µl of the previous reaction to 2 µl DEPC treated H₂O and 1 µl (300 ng) random hexamer (Invitrogen) and incubating at 70 °C for 10 min before chilling on ice. After chilling, 2 µl 5 X First Strand Buffer, 2 µl PCR Nucleotide Mix (40 mM, Promega), 1 µl 0.1 M DTT and 1 µl MMLV Reverse Transcriptase (Invitrogen) was then added and reaction mixes incubated at 25 °C 10 min, 37 °C 1 h and 70 °C 15 min. The subsequent synthesized cDNA was then diluted back to a ratio of 1:36.

2.2.13 Taqman Real Time PCR

cDNA, synthesised as described in the previous Section, was used as the template for Taqman RT-PCR. For this particular Taqman reaction, two primers and a probe are required, as shown in Figure 2.1. The primers (Applied Biosystems) are designed to bind to a particular sequence of the cDNA, which encodes the gene of interest. The probe (Applied Biosystems) is also specific for a target cDNA sequence and is labelled with a 5' 6-FAM (6-carboxyfluorescein) reporter dye and a 3' TAMRA (tetramethylrhodamine) quencher dye. Together, the primers amplify the region in the gene of interest, in doing so dislodge or cleave the probe from the DNA. The cleavage results in the reporter dye being separated from the quencher dye allowing it to emit fluorescence which can then be measured. The amount of fluorescence is directly proportional to the amount of product generated by the PCR, and to the cycle

at which the fluorescence reaches a threshold where it is significantly higher than background. This threshold can be converted to the value “counts to threshold” or C_T value. The C_T value characterises the reaction.

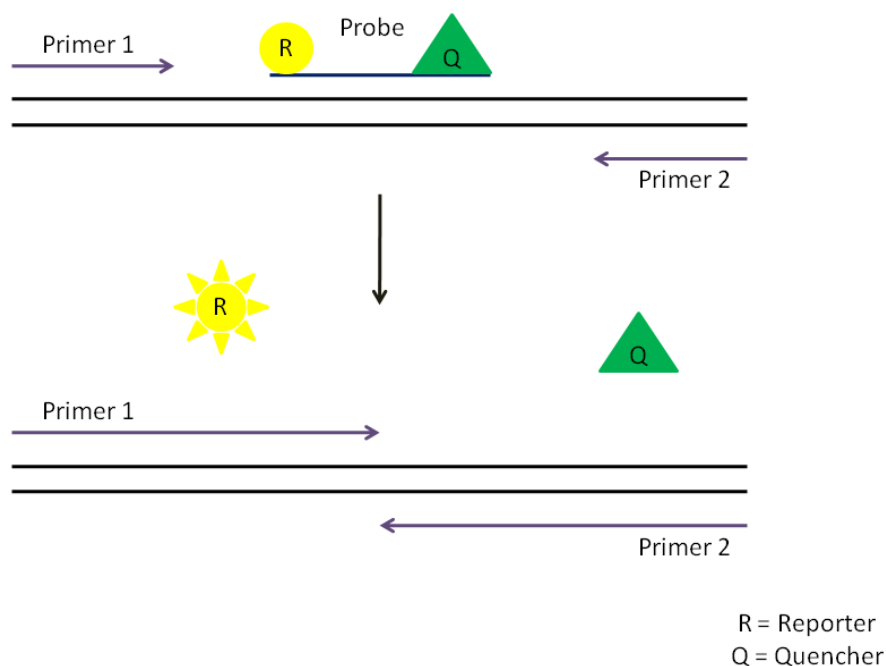


Figure 2.1: Representation of the Real Time Taqman PCR reaction

The probe consists of a reporter (R), in this case FAM and a Quencher (Q), TAMRA. The primers and probe attach to their target sequences on the cDNA. When initiated, the polymerase chain reaction allows the primers to synthesise a new copy of the DNA, in doing so, the 5' nuclease activity of Taq DNA polymerase cleaves off the probe and quencher, allowing the probe to fluoresce in the absence of the quencher.

Taqman reactions were carried out in MicroAmp optical 96 well reaction plates.

Each well contained 0.6 μ l Taqman Gene Expression Assay 20 X Mix (consisting of primers and probe, Applied Biosystems), either for *PLK1* or *β -ACTIN*, (see Table 2.4) 6 μ l Taqman 2 X Universal PCR Mix (Applied Biosystems), made up to 10 μ l

with DEPC treated H₂O and 2 µl cDNA. Amplification and analysis were carried out in an ABI PRISM 7700 Sequence Detector (Applied Biosystems). The PCR reaction itself was carried out as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 sec followed by 60 °C for 1 min. All reactions were performed in triplicate and to check there was no contamination. Control reactions containing no DNA were carried out alongside the other samples in separate wells. The probes used are given below in Table 2.4.

Gene Target of Primers/Probe	Number
PLK1	00983229_m1
<i>β-ACTIN</i>	Hs99999903_m1

Table 2.4: Gene expression Assays used in Taqman Real Time PCR

2.2.14 Chromatin Immunoprecipitations (ChIP)

Chromatin Immunoprecipitations involve immunoprecipitation of protein/DNA complexes. This involves the conjugation of an antibody to a protein which is bound to a proposed sequence of DNA, as shown in Figure 2.2. The method, initially involves the cross linking of DNA or protein/DNA complexes in the cell. Once cross linked, the cells can then be collected and lysed. Following this the sample is sonicated to further lyse the cells and to shear the DNA to produce fragments smaller than 1 Kb. Subsequently the sample can then be used in an immunoprecipitation reaction. The protein of interest together with any associated proteins are then indirectly bound to synthetic beads that recognize and bind to the antibody. The

beads are then serially washed allowing purification of the bead and protein complex. Once purified, the protein and any bound DNA is eluted. This is followed by the reversal of the cross linking of protein to DNA and the DNA subsequently purified ready for amplification, either by Polymerase Chain Reaction (PCR) or Taqman Real Time PCR.

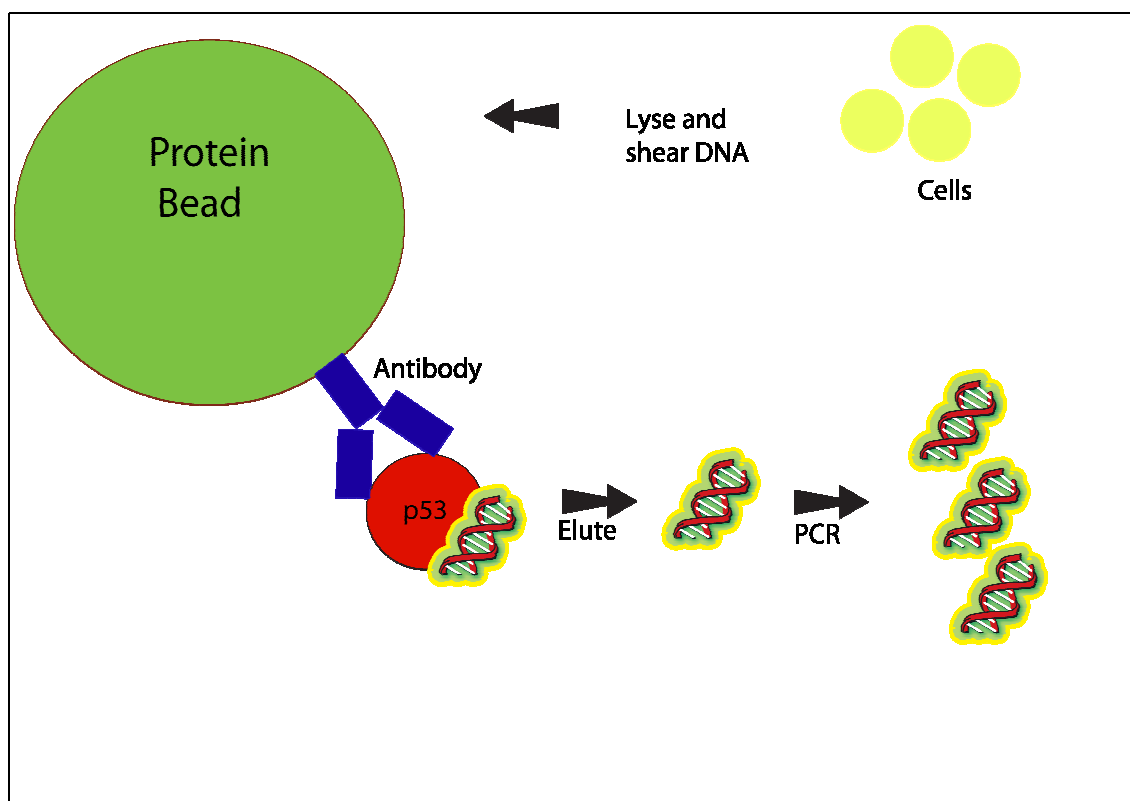


Figure 2.2: Representation of Chromatin Immunoprecipitation

Chromatin Immunoprecipitations require an antibody that binds specifically to the protein of interest. The protein binds specific DNA sequences, and the whole complex can be purified by binding the antibody to protein A beads. Once purified, the complex is disrupted and the DNA itself purified. The DNA can then be amplified up using PCR.

Cells (U2OS) were seeded at 2×10^6 per 10 cm plate and maintained in the incubator overnight at 37 °C. Etoposide (100 μ M final concentration) and Nutlin-3 (10 μ M) were added for 6 h (for each treatment, 3 plates were treated), after which the medium was removed and the cells cross linked by adding 1.5 % (w/v) paraformaldehyde for 10 min. The plates were subsequently washed with cold 1 X PBS and cells scraped into ChIP Collection Buffer (see Section 2.1 Reagents and Buffers). Cells were centrifuged at 1000 rpm for 10 min at 4 °C, the supernatant was removed and the cell pellet lysed in 150 μ l ChIP Lysis Buffer. Lysates were incubated on ice for 10 min. In order to fully lyse the cells and reduce the length of the DNA to below 1Kb the lysates were sonicated for 20 sec at 10 microns, 7 times each, leaving 1 min on ice in between each sonication to prevent the overheating and DNA denaturation. A small sample of the lysate was run on a gel to check sonication efficiency, which if efficient should yield DNA of fragments of around 200-1000 bp DNA. The lysate was then centrifuged and supernatant diluted 7-fold into ChIP Dilution Buffer, 500 μ l of lysate was used for each immunoprecipitation. In order to remove any unspecific DNA, the lysate was pre-cleared with 20 μ l of a 50 % (w/v) slurry of Protein A beads and Salmon testes DNA (2 μ g) on a rotating wheel for 2 h at 4 °C. After this, the lysate was then centrifuged at 2000 rpm for 3 min and 400 μ l of the supernatant removed into a fresh tube, to which 3 μ g anti-p53 (DO-1) antibody was added overnight at 4 °C. The remaining 100 μ l of lysate was stored at -80 °C for use as an input sample to show overall protein and DNA in the lysate. To remove any unspecific binding of proteins and/or DNA, the beads were then serially washed with ChIP Low Salt Buffer, ChIP High Salt Buffer, ChIP LiCl Buffer (as described in the Buffers and Reagents Section) and twice with 1 X TE on a rotating wheel at 4 °C for

5 min then centrifuged at 4 °C, 2000 rpm 3 min. The protein/DNA complexes were then eluted from the beads by the addition of 50 µl 1 % (w/v) SDS/0.1 M NaHCO₃ for 15 min on a rotating wheel at room temperature. This step was repeated so that there was a final volume of 100 µl. Reversal of cross linking of the samples and inputs was achieved by adding 4 µl 5M NaCl and 1 µl RNase A and placing in a water bath at 65 °C overnight. The next day DNA was recovered by adding 4 µl 1M Tris pH 6.5, 2 µl 0.5M EDTA pH 8 and 2 µl of 10 mg/ml Proteinase K and placing samples in a water bath at 45 °C for 1 h. Finally DNA was purified from the sample using the QIAquick gel extraction kit (Qiagen) and stored at -20 °C. PCR was performed on the DNA and is described in 2.4.16.

2.2.15 Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assay is an in vitro technique used to identify proteins binding to specific DNA sequences or oligonucleotides which are labeled with, for example, ³²P. When the DNA is bound to a protein, the complex moves slowly through the gel compared to the uncomplexed DNA and an overall shift can be seen when the gel is exposed to X-ray film. To further identify the protein which has bound to the DNA, an antibody against the protein can be used. This will cause a further shift in the gel which can be seen when the gel is exposed to X-ray film.

The first step of the EMSA was to anneal the pairs of oligonucleotides together into double stranded DNA (see Table 2.5). This was done by boiling the oligonucleotides (50 µl 100 pmol/µl), with 100 µl 10 X TE, 25 µl 4 M NaCl and 775 µl ddH₂O at 100

°C for 10 min and then cooling to room temperature. After this reaction, double stranded DNA is 5 pmol/μl.

The DNA was then labelled with ^{32}P by adding the DNA (2 μl, 10 μg/μl) to 1 μl 10 X Buffer for T4 Polynucleotide Kinase (Promega), 7 μl [γ - ^{32}P] ATP (0.37 Mbq/μl) and 1 μl diluted T4 PNK (10 U/μl) and incubating at 37 °C for 30 min. The sample was then run on an 8 % TBE gel for 30 min at 150 V. Once run, the gel was then exposed to Fuji-Super RX Medical X-ray film and developed in a Fuji film processor. The position of DNA was marked on the gel and the marked site was excised from the gel. The gel slice was then incubated in 0.5 ml 1 X TE at 37 °C overnight. The next day the tube was centrifuged and the supernatant containing the labelled DNA was transferred into a fresh tube and stored at -20 °C.

The binding reaction of the EMSA was assembled by adding 2 μl ^{32}P labelled DNA to 2 μl EMSA p53 5 X Binding Buffer (see Section 2.1 Reagents and Buffers), 1 μl purified p53 protein (generously supplied by Prof Ted Hupp, University of Edinburgh), 1 μl PAb421 antibody, which is a specific antibody for p53, and 4 μl ddH₂O. Reactions were incubated at 4 °C for 20 min followed by 10 min at room temperature. The bound samples were then run on a 4 % polyacrylamide gel for 2 h 30 min at 150 V at 4 °C, the gel was then vacuum dried and exposed to Fuji-Super RX Medical X-ray film and developed.

Name and pairs of Oligo- nucleotides	Sequence
PLK1 for PLK1 rev	GCCGGGCATGGTGGTGCATGCTTGTA TACAAGCATGCACCACCATGCCCCGGC
PLK1mut for PLK1mut rev	GCCGGGCATGGTGGTGGATTGTTGTA TACAACAATCCACCACCATGCCCCGGC
PG for PG rev	AGCTTAGACATGCCTAGACATGCCTA AGCTTAGGCATGTCTAGGCATGTCTA
TL for TL rev	TATGTCTAAGGGACCTGCGGTTGGCATTGATCTTG GTGCCAAGATCAATGCCAACCGCAGGTCCCTTAGACA

Table 2.5: Sequences of oligonucleotides used in EMSA.

2.3 Protein Biology Techniques

2.3.1 SDS PAGE Analysis of Proteins

Analysis of proteins by SDS-PAGE was carried out using the method of Laemmli (Laemmli, 1970) with a vertical Slab Electrophoresis Chamber apparatus (Atto) and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Running Buffer (1 X). The resolving gel contained 382 mM Tris-HCl, pH 8.8, 0.1 % (v/v) SDS, 0.1 % (v/v) ammonium persulfate, plus acrylamide added to the desired concentration (7.5 % - 15 %) from a stock of 30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide (Severn Biotech Ltd.). Polymerisation was initiated by the addition of 0.7 µl/ml N,N,N',N'-tetramethylethylenediamine (TEMED). The resolving gel was overlaid with a stacking gel consisting of 123 mM Tris-HCl, pH 6.8, 1 % (v/v) SDS, 0.1 % (v/v) ammonium persulfate, 4.9 % (v/v) acrylamide and 1.16 µl/ml TEMED. Protein samples containing 5 X SDS sample buffer were incubated at 100 °C for 3 min and centrifuged at 14000 rpm for 30 sec. Samples were loaded onto the gel and subjected to electrophoresis at 120 V until the samples passed into the resolving gel, where the voltage was increased to 180 V and run until the dye front had reached the bottom of the resolving gel. 5 µg of Pageruler Prestained Protein Ladder (Fermentas) was loaded on the gel, along with the samples, to estimate the molecular weight of proteins.

2.3.2 Western blotting

Following the separation of proteins by SDS-PAGE, proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham) using either semi-dry blotting apparatus (Biometra) or wet transfer blotting apparatus (Bio-Rad) in the appropriate Western transfer buffer. Semi-dry transfer was carried out at 200 mA for 45 min and wet transfer was carried out at 25 mA overnight or 1 h at 200 mA. Subsequently the membrane was blocked for 1 h at room temperature (RT) in Western blocking buffer and then incubated overnight at 4 °C with the primary antibody (See Table 2.6).

The membrane was washed for 3 x 5 min in Western blocking buffer, then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (see Table 2.7) in Western blocking buffer for 1 h at RT. Subsequently the membrane was washed for 1 x 5 min in Western blocking buffer and 3 x 5 min in Western washing buffer, before the addition of enhanced chemiluminescence reagents (See Section 2.1 Reagents and Buffers). Equal volumes of ECL solution 1 and ECL solution 2 were mixed together and applied uniformly to the membrane for 1 min. Excess solution was removed from the membrane and the membrane exposed to Fuji-Super RX Medical X-ray film and developed in a Fuji film processor.

Antibody	Species	Supplier	Epitope	Western Blot Concentration
Cyclin A	Rabbit	Abcam	aa 26-144	1:1000
MDM2 (SMP14)	Mouse	Santa cruz	aa 154-167	1:2000
MDM2 (4B2)	Mouse	Moravian	N-terminus	1:2000
p53 (DO-1)	Mouse	Santa cruz	aa 11-25	1:1000
p21 (H-164)	Rabbit	Santa cruz	C-terminus	1:1000
PLK1	Rabbit	Cell Signalling Technology	C-terminus	1:1000
PLK1	Mouse	Zymed	aa402-603	1:1000
phospho-Histone 3	Rabbit	Upstate	aa7-20	1:5000
acetyl-Histone 3	Rabbit	Upstate	aa 1-20	1:5000
PARP (Cleaved)	Mouse	AbDSerotec	C-terminus	1:5000
14-3-3	Goat	Santa cruz	C-terminus	1:1000
β-Actin (20-33)	Rabbit	Sigma	aa 20-33	1:5000

Table 2.6: List of primary antibodies used in Western blotting

Antibody	Species	Supplier	Western Blot Concentration
Anti Mouse Horse Radish Peroxidase Conjugated	Mouse	Santa Cruz	1:2000
Anti Rabbit Horse Radish Peroxidase Conjugated	Goat	Santa Cruz	1:5000
Anti Goat Horse Radish Peroxidase Conjugated	Donkey	Santa Cruz	1:1000

Table 2.7: List of secondary antibodies used in Western blotting

2.4 DNA Purification, Amplification and Manipulation Techniques

2.4.1 Preparation of plasmid DNA

Plasmid DNA was isolated from an overnight culture of *E.coli*, grown at 37 °C, using the PureLink Hipure Plasmid DNA purification kit from Invitrogen, Mini Preparation. A single colony of bacteria was used to inoculate 5 ml of LB, supplemented with the appropriate selective antibiotic (Ampicillin 100 µg/ml) and grown overnight at 37 °C. Following the user manual given with the kit, DNA was purified from 3 ml of the overnight culture and was eluted into 50 µl ddH₂O. Each prep gives up to 20-30 µg DNA.

In a scaled up plasmid preparation, a single colony of bacteria was used to inoculate 5 ml LB, supplemented with the appropriate antibiotic and grown overnight. 400 ml of LB, supplemented with the appropriate antibiotic was then inoculated with 1ml of the initial inoculation and grown overnight at 37 °C. The PureLink Hipure Plasmid DNA purification kit, Maxi Procedure, was used to isolate the DNA from the bacteria and DNA was eluted into 300 µl ddH₂O.

For each plasmid preparation from the large scale method, the concentration of plasmid DNA was determined by measurement of the absorbance at 260 nm (A_{260}) in an Ultraspec 2000 UV/visible spectrophotometer (Pharmacia Biotech), assuming that a solution of double-stranded DNA at 50 µg/ml has an A_{260} of 1.0. Stocks of plasmids were made at 1 µg/µl.

2.4.2 Preparation of BAC DNA

Bacterial Artificial Chromosome (BAC) DNA was isolated from an overnight culture of *E.coli*, grown at 37 °C in LB supplemented with Chloramphenicol (20 µg/ml). 3ml of the overnight culture was centrifuged at 3000 rpm for 3 min, re-suspended in 300 µl Buffer P1, and lysed by the addition of 300 µl Buffer P2. 300 µl Buffer P3 was then added and the lysate incubated on ice for 5 min. The lysate was then spun at 10,000 rpm for 10 min at 4 °C and the supernatant then transferred into a tube containing 800 µl ice cold isopropanol before further incubation on ice for a 5 min. The lysate was incubated at -80 °C for 30 min, after which the lysate was spun again at 10,000 rpm for 15 min. The supernatant was removed and 500 µl 70 % (v/v) ethanol added to the pellet and before centrifuging for 5 min, this step was then repeated. The supernatant was removed and the pellet was then air dried and re-suspended in 50 µl ddH₂O. The concentration of BAC DNA was determined by spectrometry.

2.4.3 Separation of DNA using agarose gel electrophoresis

For separating out DNA, a 1 % agarose gel was made containing 1 X TAE, agarose and ethidium bromide (400 ng/ml). Agarose Gel Loading buffer (5 X) was added to the samples which were subsequently run on a gel submerged in 1 X TAE buffer for 1 h at 100 V using a horizontal submerged agarose gel electrophoresis unit (Atto). 5 µg of a 1 Kb DNA ladder (Invitrogen) was also run on the gel in order to determine the size of the electrophoresed DNA. The DNA in the gel was then visualized using a UV-transilluminator and photographed.

2.4.4 Amplification of DNA using PCR (Polymerase Chain Reaction)

DNA was amplified by Polymerase Chain Reaction using a thermal cycler (Techne). The PCR reaction consisted of: 1 µl DNA from the preparation of BAC DNA, 10 µl 5 X Phusion GC Buffer (Finnzymes), 1 µl PCR Nucleotide Mix (Promega), 2.5 µl forward and 2.5 µl reverse primers (5 pmol each, MWG) (Table 2.8), 1 µl DMSO (Sigma), 0.5 µl (1 U) Phusion High fidelity DNA polymerase (Finnzymes) and ddH₂O up to a volume of 50 µl. The PCR cycle was as follows: Initial denaturation 95 °C 5 min, followed by 30 cycles of the following steps, denaturation at 95 °C 1 min, annealing at 59 °C 1 min, extension at 72 °C 2 min 30 secs. After the 30 cycles there was a final extension at 72 °C for 7 min.

Name of Primer	Sequence
PLK23	GAATTAAGGAGGAAGCCACCTG
PLK25	GCTCCTCCCCGAATTCAAAC

Table 2.8: Sequences of primers used in PCR reaction to amplify PLK1 promoter

2.4.5 Purification of PCR products using electrophoresis

Amplified DNA from the PCR reaction was separated using agarose gel electrophoresis and subsequently purified using electrophoresis. The band was extracted from the gel using a sterile scalpel under UV light. The gel slice was put into dialysis membrane (Size 1 12-14kDa, Medicell International) and the tubing filled with 1 X TAE. This was then electrophoresed for 1 h to elute the DNA from the gel slice and reverse electrophoresed for 1 min to remove the DNA from the

tubing. The eluted DNA was then put into a tube and 10 % (w/v) sodium acetate pH 5.4 and three times the volume of 100 % (v/v) ethanol added to it. The sample was then incubated on dry ice for 20 min, and centrifuged before re-suspending the pellet in ddH₂O.

2.4.6 Ligation of PCR DNA into plasmid

Purified PCR DNA was ligated into a plasmid vector (pSC-B), using the following reaction: 3 µl StrataClone™ Blunt Cloning Buffer, 2 µl PCR product and 1 µl StrataClone™ Blunt Vector Mix (all StrataClone), see Figure 2.3. The mixture was incubated at room temperature for 5 min and transformed into StrataClone SoloPack competent cells, as described in the next Section.

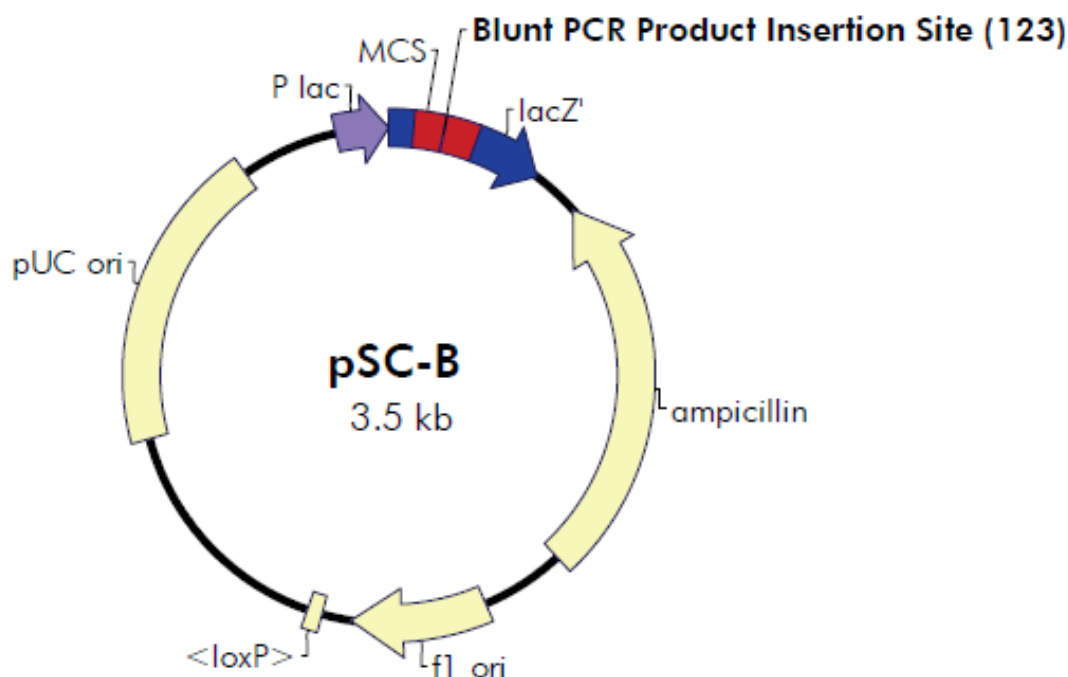


Figure 2.3: pSC-B plasmid vector from the Strataclone™ Blunt PCR Cloning Kit

The vector consists of a PCR insertion site, Ampicillin resistance gene and origin of replication site. The PCR product was inserted into the PCR insertion site. Figure taken from StrataClone Blunt Cloning Kit Manual, Stratagene.

2.4.7 Transformation of plasmid DNA into competent StrataClone SoloPack competent bacteria

StrataClone SoloPack competent bacteria (Stratagene, 50 µl) were thawed out on ice and 2 µl of ligation mix added. Cells were kept on ice for 20 min and then heat shocked at 42 °C for 45 sec before returning to ice for a further 2 min. SOC Broth was prepared from SOB Broth (see Section 2.1, Reagents and Buffers), 250 µl of which was then added to the cells which were then left to recover for 1 h at 37 °C with agitation. LB-Ampicillin (100 µg/ml) -Xgal, (5-Bromo-4-chloro-3-indoyl-β-D-

Galactopyranside, 100 µg/ml) plates were prepared and the transformation mixture spread over the plate. The plate was incubated at 37 °C overnight.

2.4.8 DNA sequencing

DNA sequencing was carried out by the DNA Analysis Facility, Human Genetics Unit, University of Dundee, Ninewells Hospital and Medical School, Dundee, Scotland.

2.4.9 Restriction enzyme digestion

Restriction digests were carried out using the appropriate restriction enzymes and buffers (NEB) which were supplemented with BSA, 100 µg/ml (NEB), if needed according to the manufacturer's instructions. 1 µg DNA was digested if the reaction was carried out for analytical purposes or 5 µg DNA was digested if the digested DNA was to be excised and purified for use in further cloning. 10 U of restriction enzyme was used in a 20 µl digestion reaction and the reaction incubated at 37 °C for 1 h. The digested DNA was run on a 1 % agarose gel and the appropriate band of DNA excised using a sterile scalpel under UV light before being purified.

2.4.10 Purification of restriction enzyme digested DNA

Restriction enzyme digested DNA was purified from the agarose gel slice using the following method. A 0.5 ml microtube was pierced with a hole in the bottom and

filled with glass fibre. The gel slice was then added to this and then incubated on dry ice for 15 min. The microtube was then placed into an eppendorf tube and centrifuged at 14000 rpm for 10 min. The microtube was discarded and the volume of supernatant was added up to 150 μ l with ddH₂O and 150 μ l butanol before a further centrifugation step. A bilayer formed with the top phase containing the butanol and ethidium bromide, which was then removed by pipette. 50 μ l 3 M sodium acetate, pH 5.4, and 1 ml 100 % ethanol was added and the sample centrifuged again. The supernatant was then removed and the pellet washed with 70 % ethanol. The ethanol was removed and the pellet, containing the DNA, was re-suspended in ddH₂O.

2.4.11 Ligation of digested DNA into the plasmid vector, pGL3

Restriction enzyme digested DNA was ligated into the pGL3-Basic Vector plasmid (Figure 2.4) (Plasmid DNA was digested with restriction enzymes that would produce complementary overhangs to overhangs created by other restriction enzymes in the insert DNA). The ligation reaction was carried out using a 1:5 molar ratio of vector to insert with 10 X ligase buffer (Promega) and 1 U of T4 DNA Ligase (Promega). The reaction was incubated at 16 °C overnight.

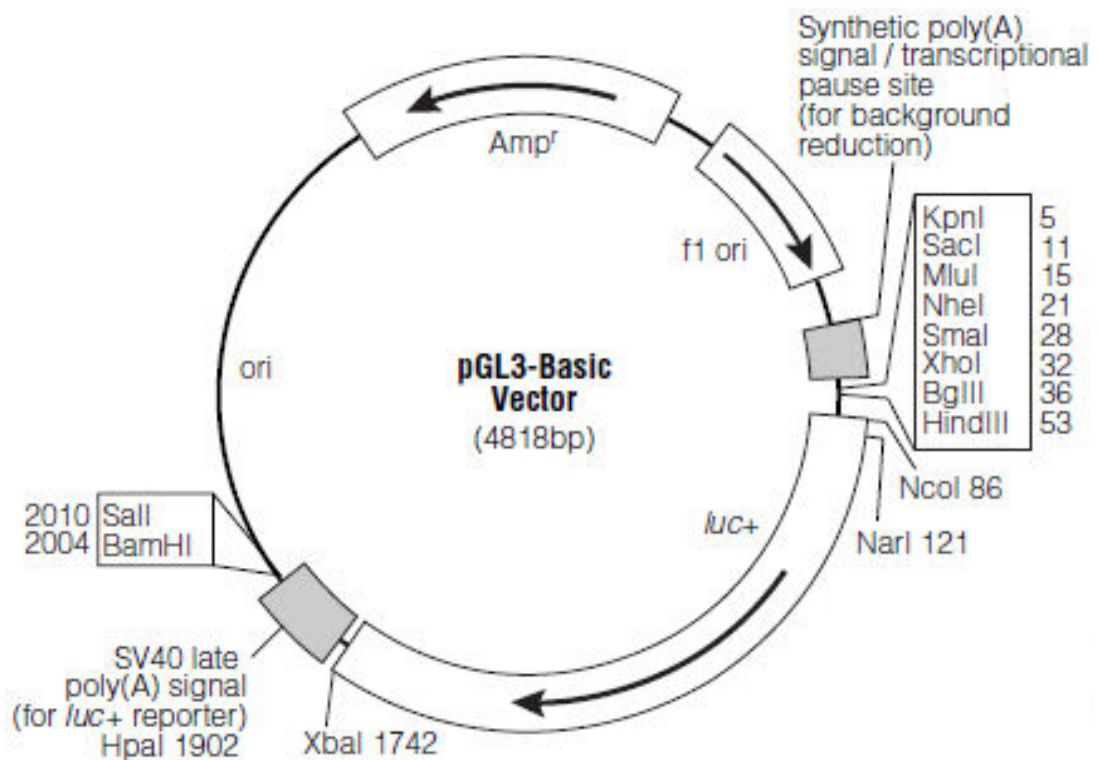


Figure 2.4: pGL3 vector (Promega)

The vector consists of an Ampicillin resistance gene, Luciferase gene and origin of replication site. The restriction enzyme digested DNA was inserted into the plasmid via two restriction digest sites situated upstream of the Luciferase gene (*Luc+*). Figure taken from pGL3 Vectors Manual, Promega.

2.4.12 Transformation of competent *E.coli* bacteria

Transformation of competent *E.coli* cells was carried out using 50 µl of DH5α competent cells. 10 µl ligation mixture, or 10 ng uncut plasmid was incubated with 50 µl cells for 30 min at 4 °C and cells then heat shocked at 37 °C for 3 min. The transformation reaction was further incubated on ice for 2 min and then incubated with 1 ml LB for 1 h at 37 °C with agitation, to allow the cells to recover. LB-

Ampicillin (100 µg/ml plates were prepared and the transformation mixture spread over the plate. The plate was incubated at 37 °C overnight.

2.4.13 Preparation of competent cells

An overnight culture of the appropriate *E. coli* strain was used to inoculate 200 ml of fresh LB and grown until the cells reached mid log phase ($OD_{600} = 0.3$). The culture was transferred to sterile centrifuge tubes and incubated on ice for 30 min. Cells were pelleted by centrifugation at 2,000 rpm for 5 min at 4 °C. The bacterial pellets were then re-suspended in 16 ml of Transformation buffer 1 (see Section 2.1 Reagents and Buffers) per 50 ml of original culture and incubated on ice for 15 min. The cells were pelleted by centrifugation as before and re-suspended in 4 ml of Transformation buffer 2 per 50 ml of original culture. The competent cells were aliquoted, snap-frozen in liquid nitrogen and stored at -80 °C.

2.4.14 Bacterial glycerol stocks

Stocks of transformed bacteria were prepared for future use and stored in 25 % glycerol at -80 °C. A single colony of transformed bacteria was grown up overnight at 37 °C in 5 ml of LB medium containing the appropriate selective antibiotic. 1 ml of the overnight culture was then mixed with 1 ml of sterile 50 % (v/v) glycerol and cells were then snap frozen on dry ice and stored at -80 °C.

2.4.15 Site Directed Mutagenesis

In order to introduce a mutation(s) to a particular sequence of the DNA in the *PLK1* promoter/pGL3-Basic Vector plasmid, QuikChange® Site Directed Mutagenesis Kit from Stratagene was used. In principle, PCR, using primers designed with the desired mutation, is carried out using the nonstrand-displacing action of the *PfuTurbo* DNA polymerase, supplied with the kit, which creates nicked circular strands. Once the PCR has been carried out, *Dpn1* which is an enzyme that digests the methylated or the parental DNA, is then added to the PCR reaction. After the parental DNA has been digested the daughter DNA can then be transformed into XL1-Blue supercompetent cells (supplied with the kit) which have the ability to repair the nicks in the mutated plasmid.

Plasmid DNA (50 ng) was added to 5 µl 10 X Reaction Buffer and 1 µl *PfuTurbo* DNA polymerase (supplied with the kit), 1.25 µl (125 ng each) of forward and reverse primers (Table 2.9), 1 µl PCR Nucleotide Mix (Promega), in a total volume of 50 µl. The PCR cycle was as follows: Initial denaturation 95 °C 4 min, followed by 18 cycles of the following steps, denaturation at 95 °C 30 sec, annealing at 65 °C 1 min and extension at 68 °C 8 min.

Name of Primer	Sequence
PLKmut1	GGGCATGGTGGTGGATTGTTGTAATTCCAGCTGC
PLKmut1rev	GCAGCTGGAATTACAACAATCCACCACCATGCCC
DMCDECHR5	GTAACGTTCCCATATCCGCGTTCACATTCGGGGAG GAG
DMCDECHR3	CTCCTCCCCGAATGTGAACGCGGATATGGGAACGT TAC

Table 2.9: Sequence of primers used to incorporate the mutation(s) into the PLK1 promoter

After PCR, 1 μ l *Dpn* I restriction enzyme, supplied with the kit, was added and incubated at 37 °C for 1 h to digest the un-mutated, template plasmid DNA. Once digested, 1 μ l of the digestion reaction was transformed into XL1-Blue supercompetent cells, supplied with the kit, by incubating the cells and DNA on ice for 15 min, heat-shocking at 42 °C for 45 sec and incubating with 1 ml LB for 1h at 37 °C with gentle agitation. LB-Ampicillin (100 μ g/ml) plates were prepared and the transformation mixture spread over the plate. The plate was incubated at 37 °C overnight.

2.4.16 Amplification of ChIP DNA using PCR

DNA from the ChIP protocol was amplified by Polymerase Chain Reaction using a thermal cycler (Techne). The PCR reaction consisted of: 2.5 μ l input DNA or 1 μ l IP DNA, 10 μ l 10 X Buffer (Promega), 1 μ l PCR Nucleotide Mix (Promega), 4 μ l forward and 4 μ l reverse primers (5 pmol each, MWG) (Table 2.10), 3 μ l MgCl₂ (Promega), 0.25 μ l (1 U) Flexi GoTaq polymerase (promega) and ddH₂O up to a volume of 50 μ l. The PCR cycle was as follows: Initial denaturation 95 °C 4 min,

followed by 30 cycles of the following steps, denaturation at 95 °C 30 sec, annealing at 60/62 °C (*p21* and *PLK1* promoter primers respectively) 1 min, extension at 72 °C 45 secs. After the 30 cycles there was a final extension at 72 °C for 5 min. Samples were subsequently run on a non-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (non-SDS-PAGE) gel and subjected to electrophoresis with non-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (non-SDS-PAGE) Running Buffer (1 X) at 120 V until the samples passed into the resolving gel, where the voltage was increased to 180 V and run until the dye front had reached the bottom of the resolving gel. 5 µg of a 1 Kb DNA ladder (Invitrogen) was also run on the gel in order to determine the size of the electrophoresed DNA. The DNA in the gel was then visualized using a UV-transilluminator and photographed.

Name of Primer	Target of Primers	Sequence
DMplk1RE25	<i>PLK1</i> promoter	AAAACCCCGCAAGACACATT
DMplk1RE23	<i>PLK1</i> promoter	TGGTCAGAGGGAGGGAAGGT
DMplk1RE2a5	<i>PLK1</i> promoter	GAGGCCAAACAAAACCCC
DMplk1RE2a3	<i>PLK1</i> promoter	TGGACACTCAGTTTCTTCCTCA
DMp215	<i>p21</i> promoter	GTGGCTCTGATTGGCTTTCTG
DMp213	<i>p21</i> promoter	CTGAAAACAGGCAGCCCAAG

Table 2.10: Sequences of primers used for PCR amplification of DNA from ChIP protocol

2.4.17 Other plasmids

pCDNA3 empty vector was provided by the lab of Professor Steve Keyse, bug stock number 439. Wild type human p53 full length in pCDNA3 was provided by Dr Nico

Dumaz, bacterial stock number 715. SV-*Renilla* Luciferase vector was provided by Dr Jean-Christophe Bourdon, bacterial stock 1427. p53 mutant his175ala in pCDNA3, was provided by Dr Jean-Christophe Bourdon and p53 mutant his273ala in pCDNA3 was provided by Dr Mark Saville, bacterial stocks 1509 and 1510, respectively. Lastly, p53 mutant L22Q/W23S in pCDNA3 and p21 HA tagged plasmid was provided by Dr Frances Fuller-Pace, bacterial stock 1582.

Chapter 3: p53 down-regulation of PLK1

3.1 Background

The tumour suppressor, p53, is a major player in the DNA damage pathway which activates the expression of genes that promote cell cycle inhibition, senescence and apoptosis, depending on the type and level of DNA damage (Resnick-Silverman and Manfredi, 2006). Furthermore p53 mediates the repression of genes involved in cell growth and proliferation (Banerjee *et al.*, 2009, St Clair and Manfredi, 2006). Many genes regulated by p53 are gradually coming to light and this chapter aims to show that Polo-like Kinase 1 or PLK1, a crucial regulator of the cell cycle, is down-regulated by p53.

In this chapter the down-regulation of PLK1 by p53 was investigated by using drug treatments and other approaches in established cell lines including HCT116 cells. HCT116 cells are a well characterised cell line originating from a human colon cancer tumour (Bunz *et al.*, 1998). Of significant value, p53-null and p21-null derivatives of these cells are available and accordingly, are ideal cell lines to use in this study as they allow the contribution of p53 and p21 to be assessed (Bunz *et al.*, 1998, Bunz *et al.*, 1999). The drug treatments utilised vary in terms of their mechanisms of action and allow the effects of p53 activation through different pathways to be assessed. Furthermore, they could potentially reveal differences in the mechanisms of PLK1 down-regulation depending on the pathways that have been activated.

The down-regulation of PLK1 could occur at transcriptional or post-translational levels and so for this reason mRNA levels have also been considered in this chapter. Moreover, repression involves a variety of other proteins and pathways, as discussed in chapter 1, therefore this chapter has assessed the involvement of these.

3.2 Aims

The aim of this chapter is to determine whether repression of Polo-Like Kinase (PLK1) occurs via the tumour suppressor, p53.

3.3 Induction of p53 levels by Etoposide results in the decrease of PLK1 levels

As discussed in Chapter 1, Etoposide is a potent activator of the p53 pathway and induces expression of p53. Figure 3.1 (A) shows the increase of p53, after the addition of Etoposide to HCT116 cells, over a 24 h period. This is concomitant with an increase of its downstream target, p21, as expected. Notably, this is also concomitant with a decrease in PLK1. As a control β -Actin has also been probed to show that the protein levels are equal in each lane. Figure 3.1 (B) shows a titration of increasing concentrations of Etoposide on HCT116 cells. The results demonstrate p53 is induced by Etoposide in a concentration-dependent manner concomitantly with increasing p21 protein levels. In contrast, PLK1 protein levels decrease concentration-dependently.

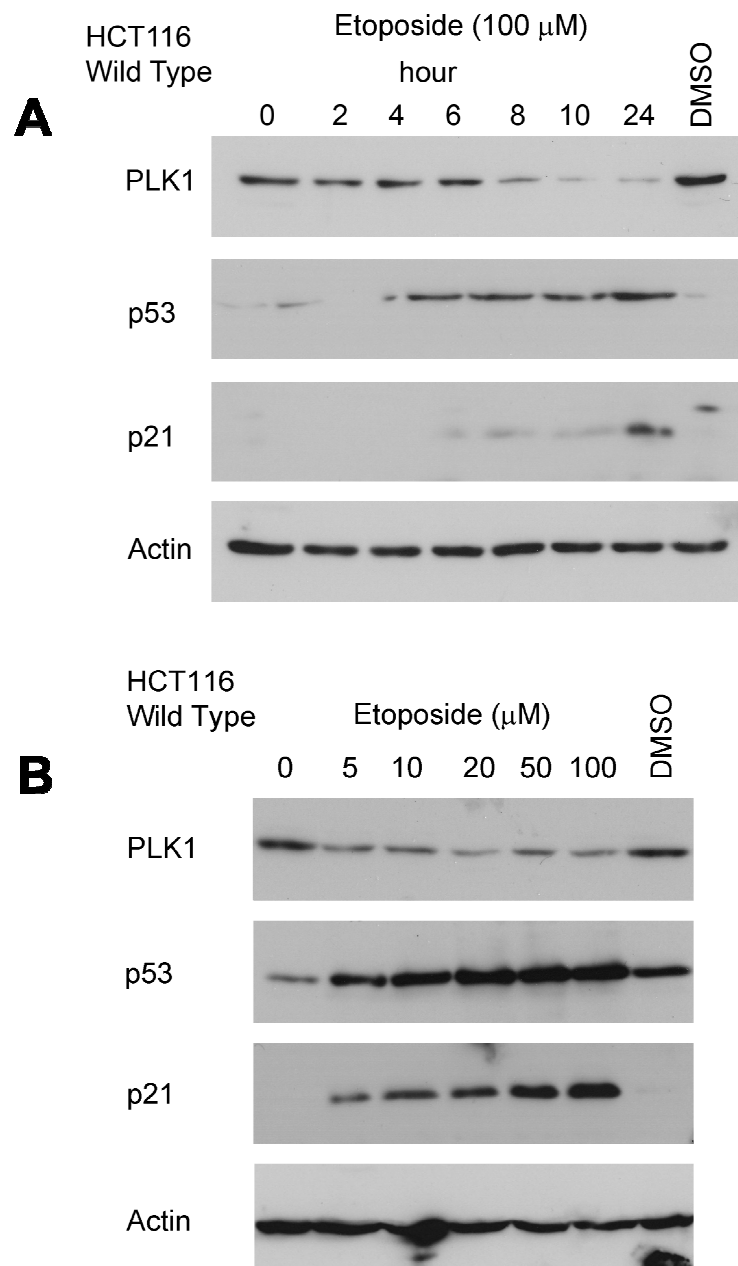


Figure 3.1: PLK1 down-regulation is concomitant with increasing levels of p53 and p21 after Etoposide treatment in HCT116 wild type cells

A) HCT116 wild type cells were treated with 100 μ M Etoposide and lysed at 2 h intervals over a 10 h period with a final time point lysed at 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments. **B)** HCT116 wild type cells were treated with increasing concentrations of Etoposide for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 2 independent experiments.

These results confirm that Etoposide induces the p53 pathway in HCT116 cells. The decrease in PLK1 levels suggests the possibility that the p53 pathway is down-regulating protein levels of PLK1.

To determine whether the decrease in PLK1 protein levels is dependent on p53, additional experiments were carried out in parallel in the p53-null derivative of HCT116. As shown in Figure 3.2 (A) in the Etoposide time course and in the Etoposide titration (B), the levels of PLK1 protein do not decrease in the absence of p53. The lack of induction of p21 confirms the absence of a functional p53 pathway. Positive controls that were run alongside the p53-null cell lysates confirm that the p53 and p21 proteins could be detected by the Western blotting procedure.

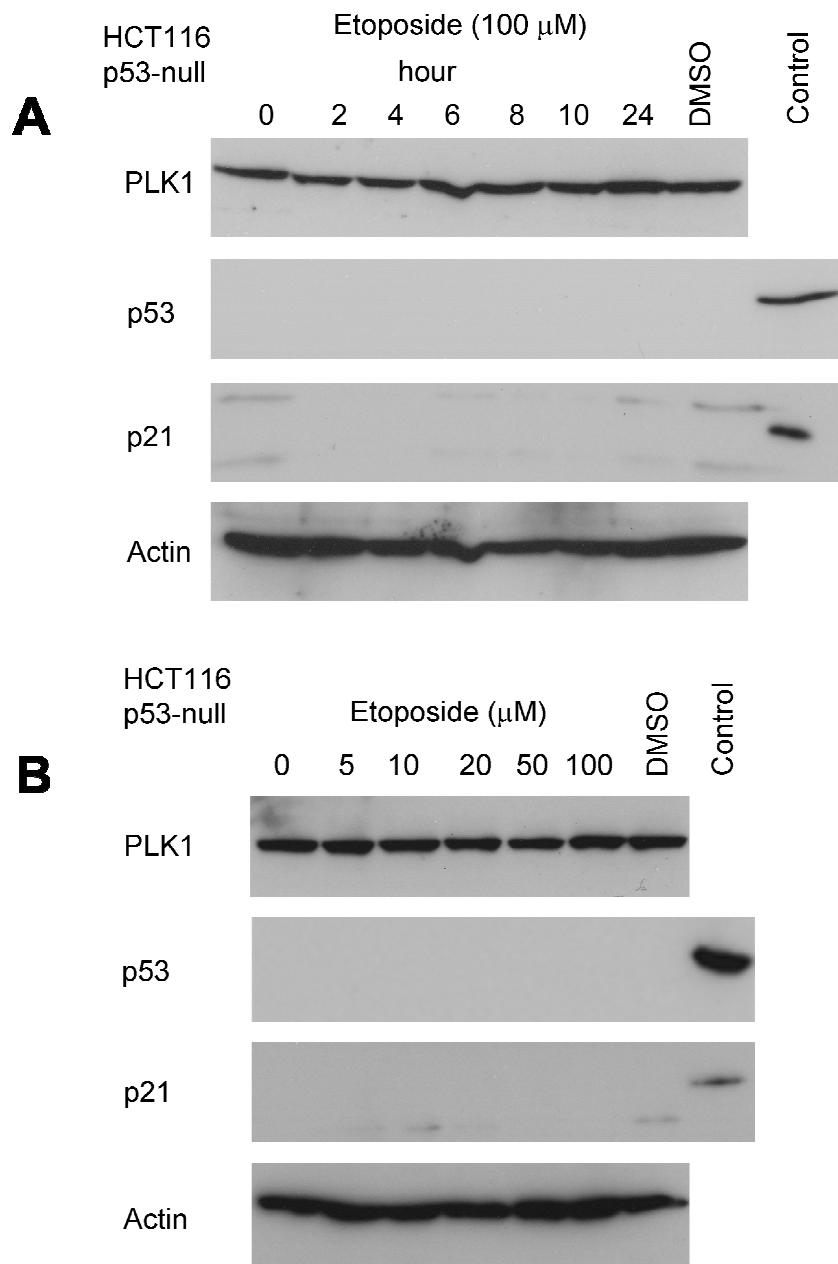


Figure 3.2: PLK1 down-regulation is absent after Etoposide treatment in HCT116 p53-null cells

A) HCT116 p53-null cells were treated with 100 μ M Etoposide and lysed at 2 h intervals over a 10 h period with a final time point lysed at 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments. **B)** HCT116 p53-null cells were treated with increasing concentrations of Etoposide for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 2 independent experiments.

In conclusion these data show that Etoposide induced reduction in PLK1 protein is dependent on the presence of p53.

PLK1 levels are regulated in a cell cycle dependent manner in many cell lines and are correspondingly low throughout G1 and S phase but increase throughout the G2 phase and peak at mitosis (Hamanaka *et al.*, 1995). This effect is mediated by transcriptional repression through the promoter element termed CDE/CHR, located within the core region of the PLK1 promoter (Uchiumi *et al.*, 1997). Given that p53 can arrest cells at the G1/S boundary by inducing p21 (Waldman *et al.*, 1995), the decrease in PLK1 levels described above could simply reflect p53-dependent G1 arrest. Alternatively, p53 itself or a downstream target of p53 could mediate the repression of PLK1 expression: for example, p21 is known to repress the expression of genes such as CDC25C (St Clair and Manfredi, 2006). Before testing these possibilities it was necessary to check whether PLK1 levels are indeed cell cycle-dependent in our experimental system.

3.4 PLK1 protein levels are regulated through the cell cycle

To determine whether PLK1 levels change throughout the cell cycle, a double Thymidine block and release experiment was carried out using U2OS and HCT116 cells. Thymidine has a negative feedback effect on nucleotide biosynthesis and can therefore prevent cells from entering S phase. The removal of the Thymidine then allows the cells to enter S phase and proceed throughout the cell cycle synchronously. A “double” Thymidine block ensures that the cells are as synchronous as possible. To check the synchrony of the cells, Western blots showing phosphorylated histone 3 (p-H3), which is a marker of mitosis (Hendzel *et al.*, 1997),

and Cyclin A, which is a marker of S/G2 phase were carried out (Pagano *et al.*, 1992). As shown in Figure 3.3, phosphorylated histone 3 levels are high, as expected at around 12 h and 14 h (M phase) and Cyclin A levels are high, as expected, between 4 h to around 10 h (S/G2 phase).

Examination of the PLK1 levels in these synchronous populations confirmed that PLK1 levels are elevated during the G2 and M phases of the cell cycle as reported for other cell lines (Golsteyn *et al.*, 1995, Hamanaka *et al.*, 1995).

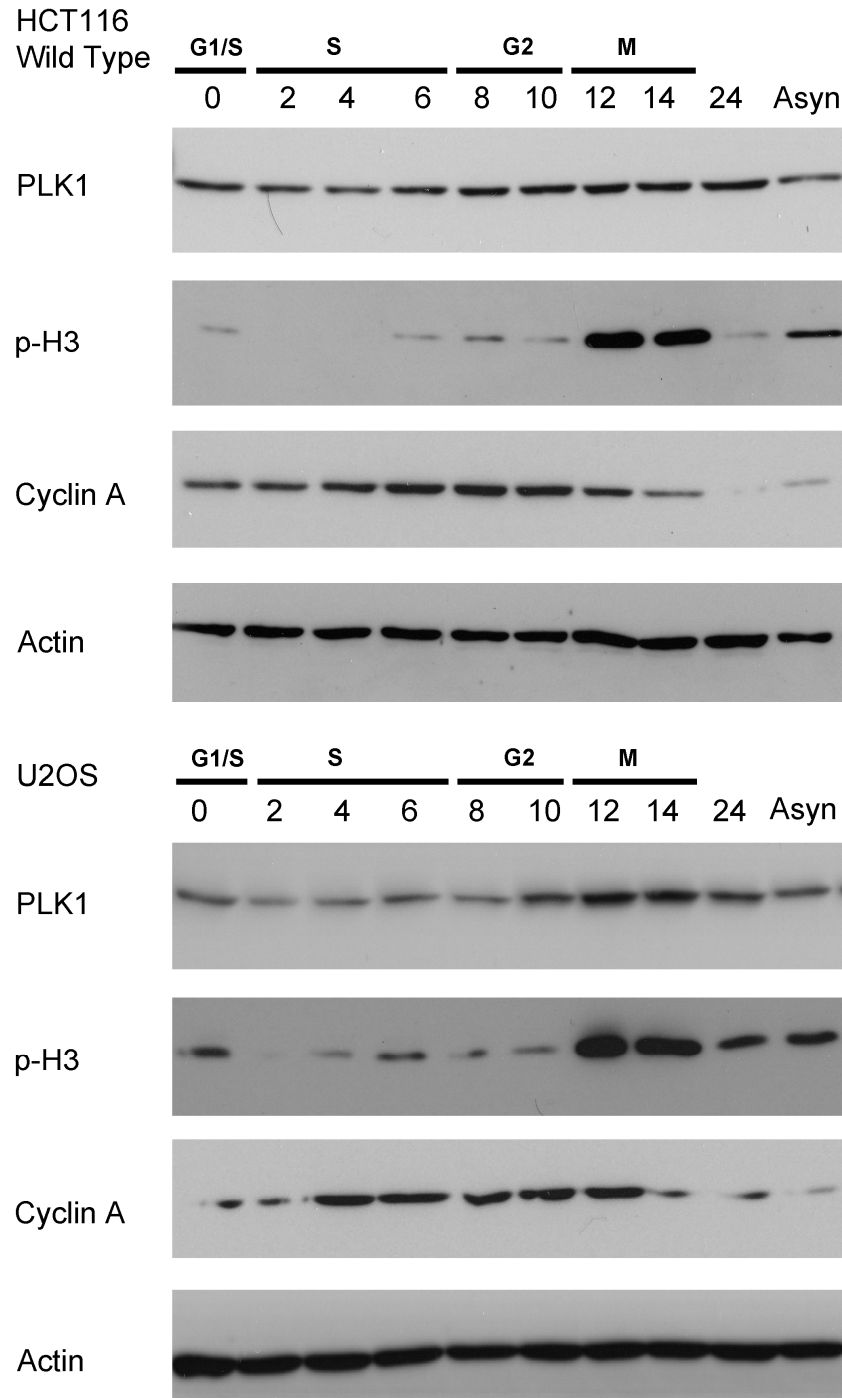


Figure 3.3: Double Thymidine block and release showing that PLK1 is regulated through the cell cycle

HCT116 wild type cells and U2OS cells were treated with Thymidine (2 mM) for 16 h and then grown for 8 h before Thymidine was added again for 16 h. After removal of Thymidine, a plate of cells was lysed every 2 h over a 24 h period and a final time point lysed at 24 h. An unsynchronised (Asyn) plate of cells was also lysed. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-pH3 antibodies, anti-Cyclin A antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments.

These data also support the possibility, as mentioned above, that the decrease in PLK1 levels observed after treatment with Etoposide simply reflects a substantial p53-mediated arrest at G1/S, a point in the cell cycle where PLK1 levels are naturally low. Our next aim was therefore to determine what effect the absence of p21 has on the down-regulation of PLK1.

3.5 PLK1 down-regulation by Etoposide is not due to G1/S checkpoint arrest

In order to test whether PLK1 can still be down-regulated in the absence of p21 (and therefore in the absence of a G1/S arrest), the p21-null derivative of HCT116 was treated, as before, with Etoposide. As shown in Figure 3.4 (A), the PLK1 protein levels decrease initially in the first 10 h. Interestingly, however, at 24 h the levels of PLK1 are higher than those in the controls lacking Etoposide or having DMSO alone. As shown in Figure 3.4 (A and B) the levels of p53 are found at higher levels after treatment with Etoposide but the p53 induction was impaired in comparison with the levels achieved in the wild type cells. The titration (B) shows that PLK1 levels increase with various concentrations of Etoposide.

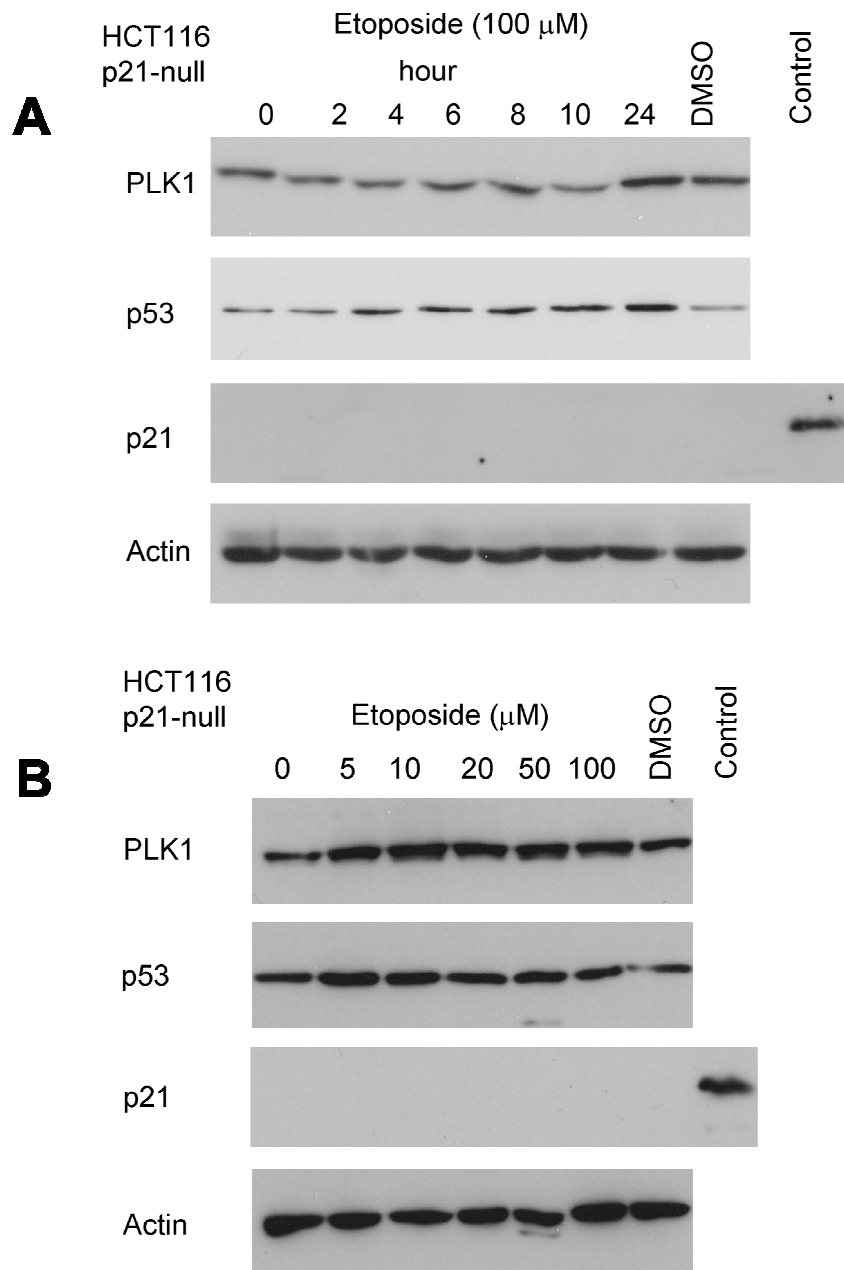


Figure 3.4: PLK1 down-regulation is concomitant with increasing levels of p53 after Etoposide treatment in HCT116 p21-null cells

A) HCT116 p21-null cells were treated with 100 μ M Etoposide and lysed at 2 h intervals over a 10 h period with a final time point lysed at 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments. **B)** HCT116 p21-null cells were treated with increasing concentrations of Etoposide for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 2 independent experiments.

These data suggest that the decrease of PLK1 levels is not dependent on p21. In order to confirm this, further experiments were carried out and are described below.

While the results from the p21-null cells suggest that the down-regulation of PLK1 could occur in the absence of this cell cycle inhibitor, it was necessary to check that Etoposide treatment did not result in a G1/S arrest in these cells. To do this, flow cytometry was carried out. In this procedure the cells are labelled with BrdU and, after harvesting, with a FITC- conjugated antibody. The cells were also labelled with propidium iodide to stain the DNA (as described in Chapter 2). The amount of DNA in each cell and the proportion of cells that were actively progressing through S phase in the time the cells were incubated with BrdU were calculated. Using these results, it was then confirmed what percentage of cells there were in each stage of the cell cycle (see Appendix).

Prior to treating the cells with Etoposide, an optimisation experiment was carried out in order to determine the most appropriate density of cells for carrying out analysis. As shown in Figure 3.5, seeding of the HCT116 parental cells at the higher density showed that there were a greater proportion of cells in G2/M. In both p53- null derivatives and p21-null derivatives, the cell cycle profiles were very similar at each density.

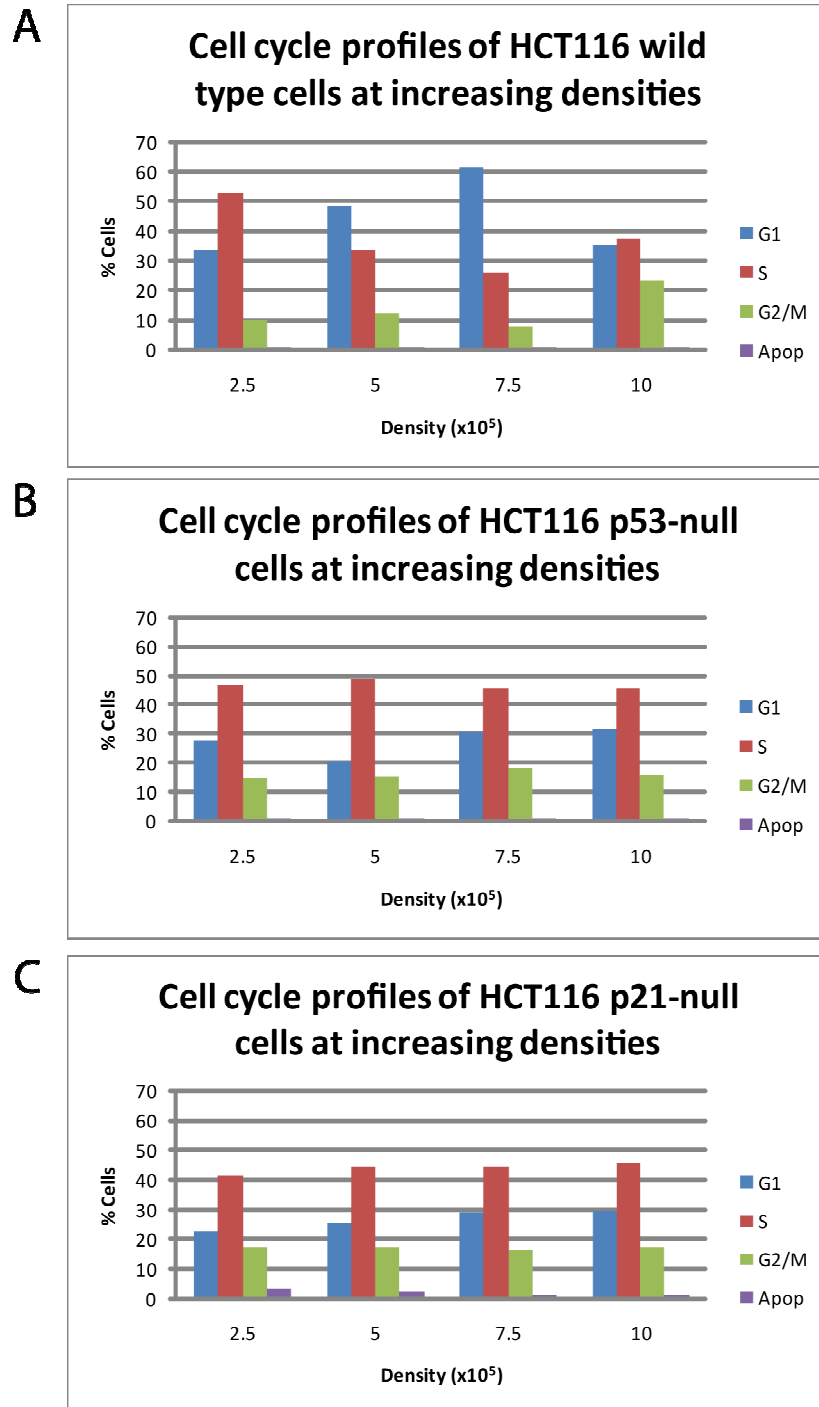


Figure 3.5: Cell cycle profiles of HCT116 wild type, p53-null and p21-null at increasing densities

This figure shows the flow cytometry data of different densities of HCT116 cells and their derivatives. Graph A shows the cell cycle profiles of the different densities of HCT116 wild type cells. Graph B, shows the HCT116 p53-null cells and Graph C, shows the HCT116 p21-null cells. These graphs are representative of 2 independent experiments.

Subsequent experiments were therefore carried out at a density of 2.5×10^5 or 1×10^6 .

To analyse whether the DNA damaging agent, Etoposide, inhibits the cell cycle at the G1/S checkpoint or G2/M checkpoint, individual plates of each cell line were treated in the absence or presence of Etoposide. Cells were then examined by flow cytometry, as described in Chapter 2 and the percentage of cells in each phase of the cell cycle, determined (see Appendix). Figure 3.6 shows that the percentage of HCT116 wild type cells in G1 phase and S phase of the cell cycle has decreased when they were treated with Etoposide. However, the percentage of cells in G2/M phase has increased. Furthermore, the addition of Etoposide in the p53-null cell derivatives decreases the percentage of cells in G1 phase. S phase cells increase slightly and the number of cells in G2/M increases. In p21-null cells the percentage of cells in G1 phase has decreased, likewise the number of cells in S phase has decreased. The number of p21-null cells in G2/M phase treated with Etoposide has increased compared to the untreated.

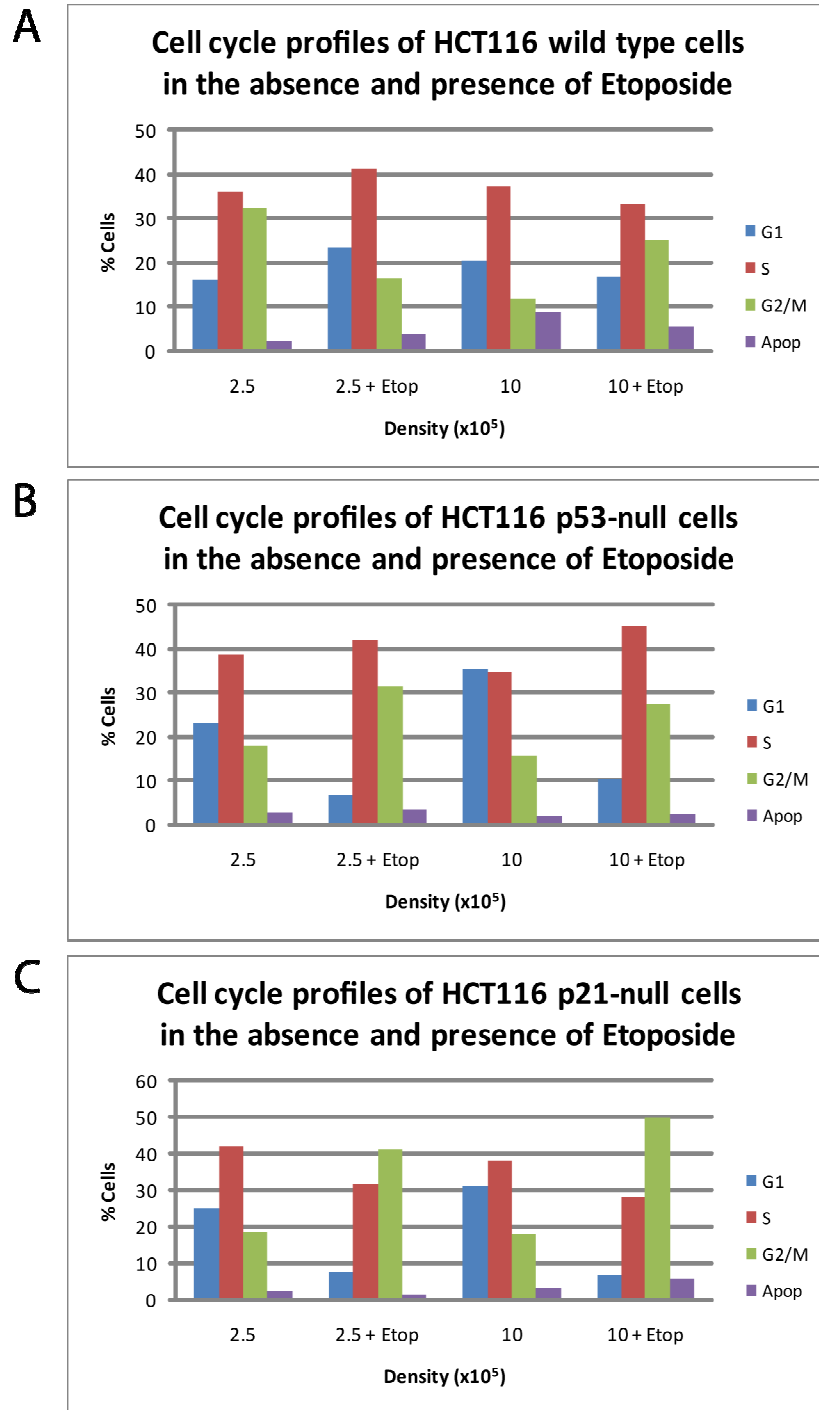


Figure 3.6: Cell cycle profiles of HCT116 wild type, p53-null and p21-null at two densities in the absence and presence of Etoposide

This figure shows the flow cytometry data of two densities of HCT116 cells (2.5×10^5 and 1×10^6) and their derivatives in the absence and presence of Etoposide ($100 \mu\text{M}$). Graph A shows the cell cycle profiles of the HCT116 wild type cells in the absence and presence of Etoposide. Graph B, shows the HCT116 p53-null cells and Graph C, shows the HCT116 p21-null cells. These graphs are representative of 2 independent experiments.

Taken together, these results show that the decrease seen in PLK1 levels when cells are treated with Etoposide is not due to an inhibition of the cell cycle at the G1/S checkpoint when PLK1 levels are low.

Interestingly, the results also show that G2/M checkpoint inhibition by Etoposide is not dependent on either p53 or p21 (Stark *et al.*, 2006, Taylor *et al.*, 2001b). Etoposide is a DNA damaging agent which activates many DNA damage pathways, including the p53 pathway. Therefore other pathways may also contribute to G2/M checkpoint arrest and arrest by Etoposide.

In order to elucidate whether p53 induction is sufficient to down-regulate PLK1 in the absence of DNA damage, similar experiments were carried out with the MDM2 inhibitor, Nutlin-3 and described in the next Section.

3.6 Induction of p53 levels by Nutlin-3 results in PLK1 down-regulation

As Nutlin-3 induces p53 and the p53 pathway in a non DNA damage dependent manner and induces cell checkpoint arrest (Vassilev *et al.*, 2004), our next aim was to determine whether down-regulation of PLK1 by Nutlin-3 is indeed dependent on p53.

To answer this, HCT116 cells and their derivatives were treated with Nutlin-3. Figure 3.7 (A), shows HCT116 wild type cells treated with Nutlin-3 over a 24 h period. As expected, p53 levels increase over time, which is concomitant with increasing levels of p21. PLK1 levels, however, decrease suggesting a role for p53 in PLK1 down-regulation. Figure 3.7 (B) shows that with increasing concentrations of Nutlin-3, p53 and p21 levels increase. As expected, PLK1 levels decrease.

This result shows that Nutlin-3 induces a p53 response which stimulates a decrease in PLK1 levels. This suggests that p53 mediates the down-regulation of PLK1 levels.

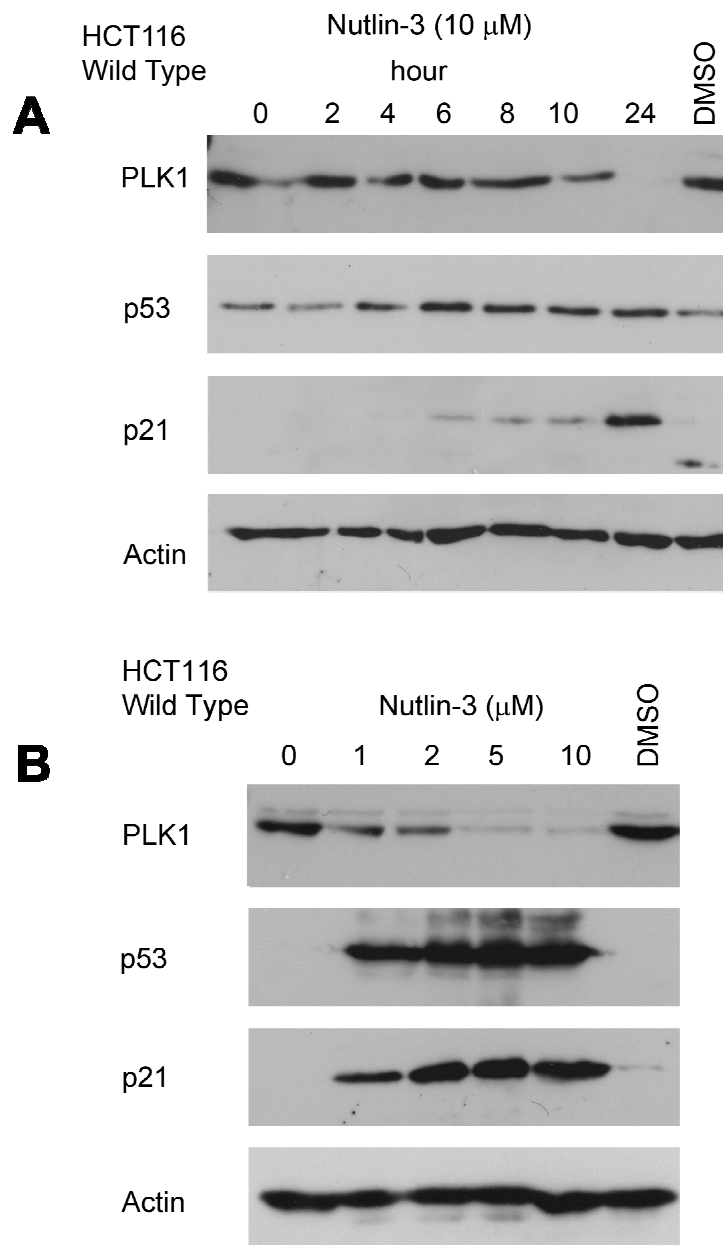


Figure 3.7: PLK1 down-regulation is concomitant with increasing levels of p53 after Nutlin-3 treatment in HCT116 wild type cells

A) HCT116 wild type cells were treated with 10 μ M Nutlin-3 and lysed at 2 h intervals over a 10 h period with a final time point lysed at 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments. **B)** HCT116 wild type cells were treated with increasing concentrations of Nutlin-3 for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 2 independent experiments.

Similar to the Etoposide experiments, HCT116 p53-null derivatives were treated with Nutlin-3 to verify whether PLK1 down-regulation was dependent upon p53 (Figure 3.8). These cells were treated with Nutlin-3 over a 24 h period (A) and they were also treated with increasing concentrations of Nutlin-3 for 24 h (B). The results show that when these cells are treated with Nutlin-3 over a 24 h period, PLK1 levels stay constant. In the absence of p53, PLK1 down-regulation is abolished. Furthermore when cells are treated with increasing concentrations of Nutlin-3, PLK1 levels stay constant. To confirm there is no p53 and therefore no p21 protein produced in this cell line, p53 and p21 Western blots were carried out, alongside positive controls for these proteins.

Results from Figure 3.7 and Figure 3.8 confirm PLK1 down-regulation is dependent on p53. However at this stage it was not clear whether PLK1 down-regulation by Nutlin-3 was mediated by p53 or induced by a downstream target of p53, such as p21. Consequently, similar to the Etoposide treatment, Nutlin-3 experiments were carried out in the HCT116 p21-null cell derivatives (Figure 3.9). Once more, HCT116 p21-null cells were treated with Nutlin-3 over a 24 h time course (A). These cells were also treated with increasing concentrations of Nutlin-3 for 24 h (B). To confirm there is no p21 protein produced in this cell line, p21 Western blots were performed, alongside positive controls for the proteins.

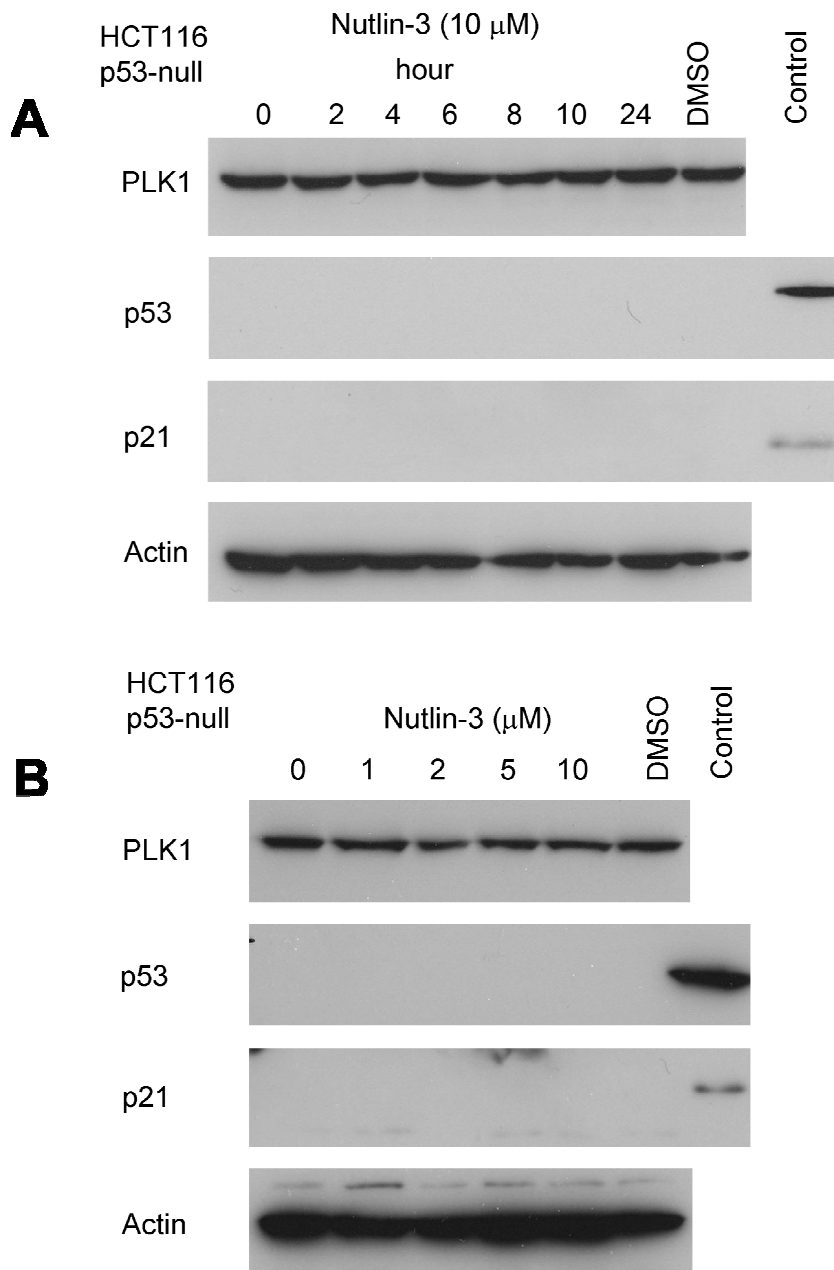


Figure 3.8: PLK1 down-regulation is not shown after Nutlin-3 treatment in HCT116 p53-null cells

A) HCT116 p53-null cells were treated with 10 μ M Nutlin-3 and lysed at 2 h intervals over a 10 h period with a final time point lysed at 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments. **B)** HCT116 p53-null cells were treated with increasing concentrations of Nutlin-3 for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 2 independent experiments.

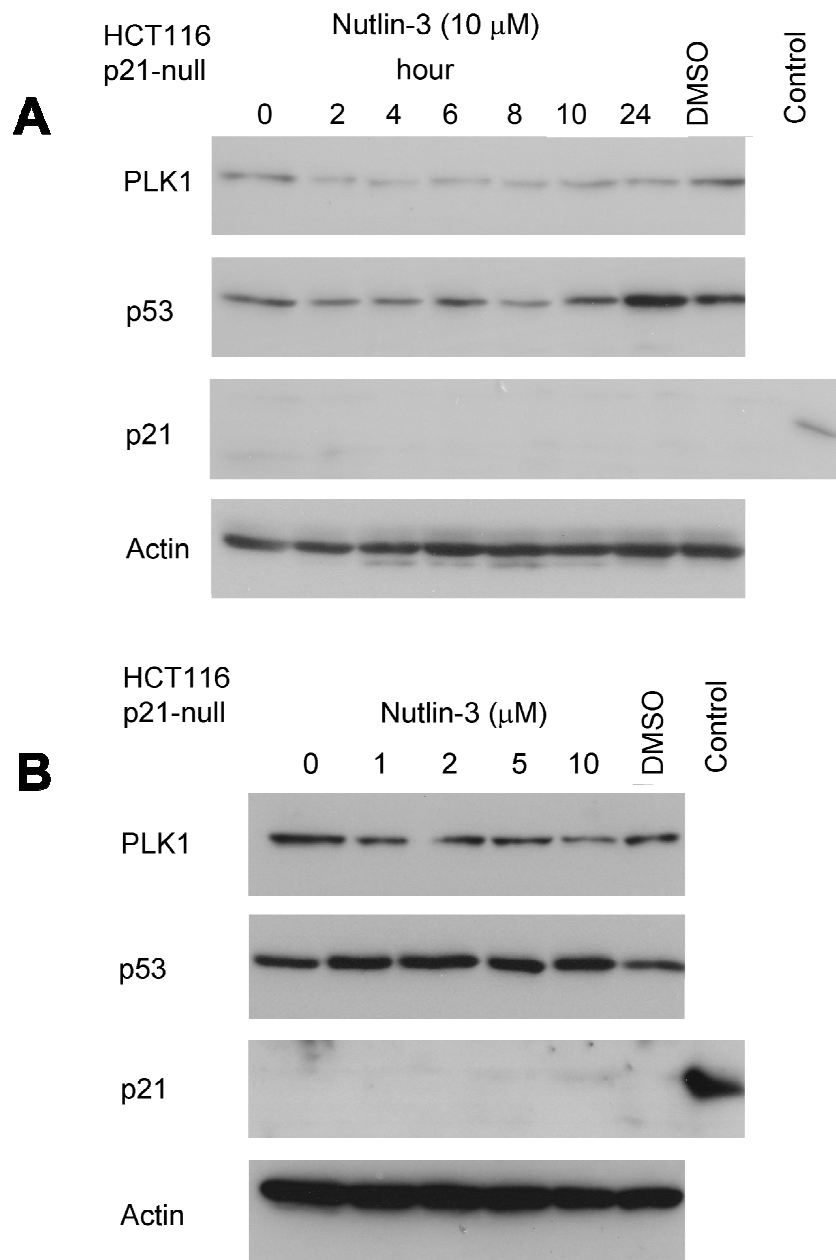


Figure 3.9: PLK1 down-regulation is concomitant with increasing levels of p53 after Nutlin-3 treatment in HCT116 p21-null cells

A) HCT116 p21-null cells were treated with 10 μ M Nutlin-3 and lysed at 2 h intervals over a 10 h period with a final time point lysed at 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments. **B)** HCT116 p21-null cells were treated with increasing concentrations of Nutlin-3 for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 2 independent experiments.

As shown in Figure 3.7, PLK1 levels decrease with the addition of Nutlin-3 and decrease with increasing concentrations of Nutlin-3. However, compared to the Nutlin-3 treated HCT116 wild type cells (Figure 3.7), PLK1 levels are not abolished at the 24 h time point in the p21-null cells. In addition to this, the p53 response is much slower, with p53 levels only increasing at the 24 h time point in the p21-null derivatives. The p53 response in this cell line, initiated by the addition of Nutlin-3, does not appear to be as high as that shown in the HCT116 wild type cells. There may be many explanations as to why the p53 response is weaker in this cell line. A possibility may be that this is due to an intrinsic factor in this particular derivative of HCT116, therefore *p21* siRNA knock-down experiments may resolve this issue, as discussed later.

3.7 PLK1 down-regulation by Nutlin-3 is not due to G1/S checkpoint arrest

Overall, results from the Nutlin-3 experiments conclude that the down-regulation of PLK1 is dependent on p53. In the absence of p21, down-regulation does occur but not to the same extent as when p21 is present. This indicates that p21 may also be involved in the down-regulation of PLK1, either through repression or simply through the arrest of cells at the G1/S checkpoint.

To investigate whether down-regulation of PLK1 by Nutlin-3 is due to a repression by p53 or p21, or simply down to G1/S checkpoint arrest, Nutlin-3 was added to all three types of cell and analysed by flow cytometry (see Appendix). As shown in Figure 3.10, there was an increase in the number of HCT116 parental cells in G1 phase and in G2/M phase of the cell cycle in the presence of Nutlin-3. However,

when HCT116 p53-null and p21-null cells were treated with Nutlin-3 there was no change to the proportion of cells in each phase of the cell cycle.

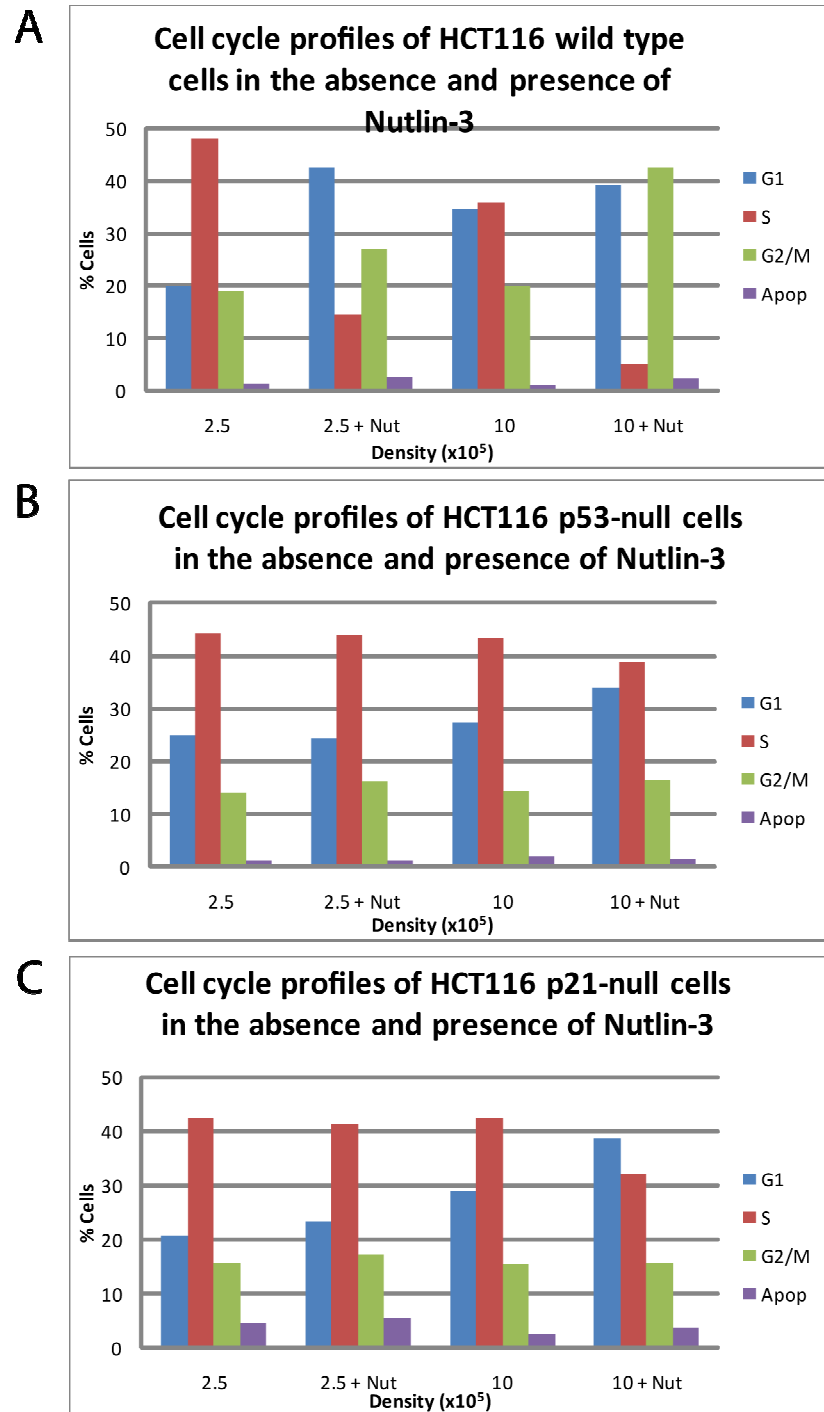


Figure 3.10: Cell cycle profiles of HCT116 wild type, p53-null and p21-null at two densities in the absence and presence of Nutlin-3

This figure shows the flow cytometry data of two densities of HCT116 cells (2.5×10^5 and 1×10^6) and their derivatives in the absence and presence of Nutlin-3 ($10 \mu\text{M}$). Graph A shows the cell cycle profiles of the HCT116 wild type cells in the absence and presence of Nutlin-3. Graph B, shows the HCT116 p53-null cells and Graph C, shows the HCT116 p21-null cells. These graphs are representative of 2 independent experiments.

In conclusion, these results show that Nutlin-3 has a different effect on the cell cycle to that of Etoposide (Figure 3.6). Nutlin-3 inhibits the cells at the G1/S phase of the cell cycle and at the G2/M phase whereas Etoposide gives a mainly G2/M arrest. Furthermore the number of cells in S phase following Nutlin-3 treatment has almost been completely abolished. Interestingly these results also show that Nutlin-3 is unable to cause cell cycle checkpoint block in the absence of p53 or p21, confirming that Nutlin-3 functions by inducing p53 and p21.

In summary, all of the data so far show that the induction of p53, by Etoposide or Nutlin-3, down-regulates PLK1. While this can occur in the absence of p21, the data suggest that p21 may contribute to this although it is unlikely that this occurs through cell cycle arrest. In addition to blocking cell cycle progression, p21 is a known repressor of certain genes and therefore it may be possible that p21 can affect the expression of PLK1. Alternatively since the induction of the p53 pathway is weaker in the p21-null cells there may be other, as yet unknown, changes in these cells other than the absence of p21, possibly the result of continuous passage. For these reasons, *p21* siRNA experiments have been carried out to determine, using an independent approach, the extent to which p21 has on the down-regulation of PLK1.

3.8 p21 plays a role in PLK1 repression

To verify whether p21 contributes to the down-regulation of PLK1, *p21* siRNA experiments were carried out in HCT116 parental cells.

In order to silence p21 expression, cells were transfected with siRNA for 48 h. However, as p21 levels are undetectable in unstimulated cells, the cells were treated in the absence or presence of Nutlin-3, 24 h after transfection with siRNA, to induce

the expression of p21. HCT116 were untransfected, mock transfected (which accounts for any affects the tranfection reagent or Optimem is having on the cells), treated with a scrambled (non-silencing) siRNA which is unspecific to any gene or cells were treated with siRNA targeted towards the p21 gene (Figure 3.11). The p21 Western blot shown in Figure 3.11, shows that the induction of p21 by Nutlin-3 occurred in the non-transfected, mock transfected and scrambled siRNA transfected cells but was not present in non Nutlin-3 treated cells, as expected. However in the Nutlin-3 treated and *p21* siRNA-transfected cells p21 could not be detected, indicating that the siRNA targeted toward the p21 gene has eliminated the expression of p21. Furthermore, to show that the p53 pathway had been activated p53 was also probed and, as revealed in the p53 Western blot, p53 is induced by the addition of Nutlin-3. This coincides with an increase in MDM2, as anticipated. In all cases PLK1 levels were depleted when the cells were treated with Nutlin-3. However, in the cells in which p21 expression had been silenced, the down-regulation of PLK1 levels was not as strong as with the control transfections.

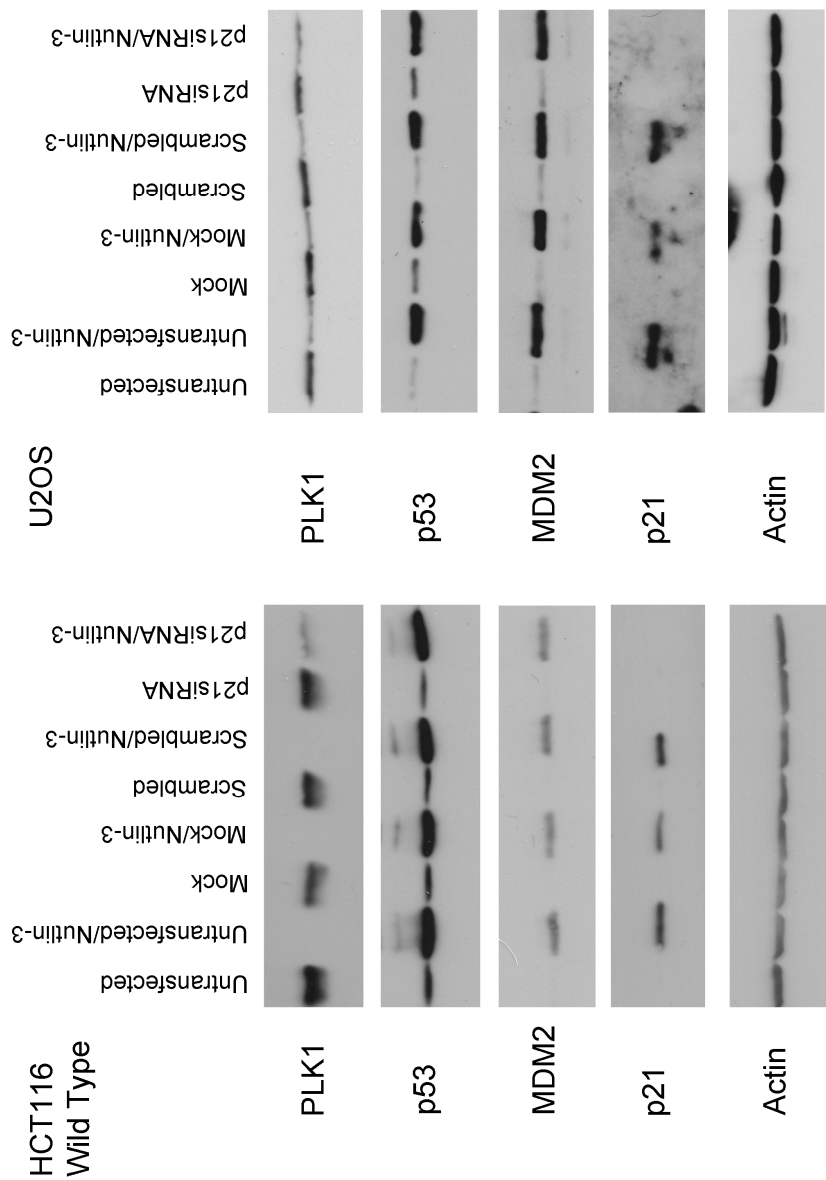


Figure 3.11: Silencing p21 by siRNA shows PLK1 by siRNA shows p21 plays a part in PLK1 down-regulation

HCT116 wild type cells were treated with OptiMem/Lipofectamine RNAiMAX only, OptiMem/Lipofectamine RNAiMAX with Scrambled siRNA (30 pMol) or OptiMem/Lipofectamine RNAiMAX with p21 siRNA (30 pMol). U2OS cells were treated with OptiMem/Lipofectamine 2000 only, OptiMem/Lipofectamine 2000 with Scrambled siRNA (100 pMol) or OptiMem/Lipofectamine 2000 with p21 siRNA (100 pMol). Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-MDM2 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments.

This result is consistent with the observations made using the HCT116 p21-null derivative when treated with Nutlin-3 and confirms that p21 can make a contribution to PLK1 down-regulation.

In addition to the HCT116 cells, the identical experiment was carried out in U2OS cells. Similar data were obtained (Figure 3.11), although the contribution made by p21 in these cells appeared to be much less significant as compared with the HCT116 cells.

In conclusion, *p21* siRNA experiments indicate that down-regulation of PLK1 is dependent, but only in part, on p21 and that this may be dependent on the cell type.

3.9 Induction of p53 levels by Cisplatin causes PLK1 down-regulation

Cisplatin is a platinum based DNA damaging agent, which, like Etoposide and Nutlin-3, is able to induce p53 and the p53 pathway (Siddik, 2003). However, Cisplatin has a very different mechanism of action in DNA damage compared to that of Etoposide, as discussed in Chapter 1.

As shown in Figure 3.12 (A), p53 levels increase slowly after the addition of Cisplatin to HCT116 wild type cells, as expected, concomitant with an increase in p21. Notably, PLK1 levels decrease slowly after the addition of Cisplatin. Figure 3.12 (B), shows levels of p53 and p21 also increase with the addition of increasing concentrations of Cisplatin. As expected, PLK1 levels decrease.

To determine whether the addition of Cisplatin would down-regulate PLK1 levels in the absence of p53, similar experiments were carried out in HCT116 p53-null cell derivatives, as shown in Figure 3.13 (A), shows PLK1 levels do not change in the

absence of p53, when cells are treated with Cisplatin for increasing periods of time. Figure 3.13 (B) also shows that PLK1 levels do not change in the absence of p53 when cells are treated with increasing concentrations of Cisplatin.

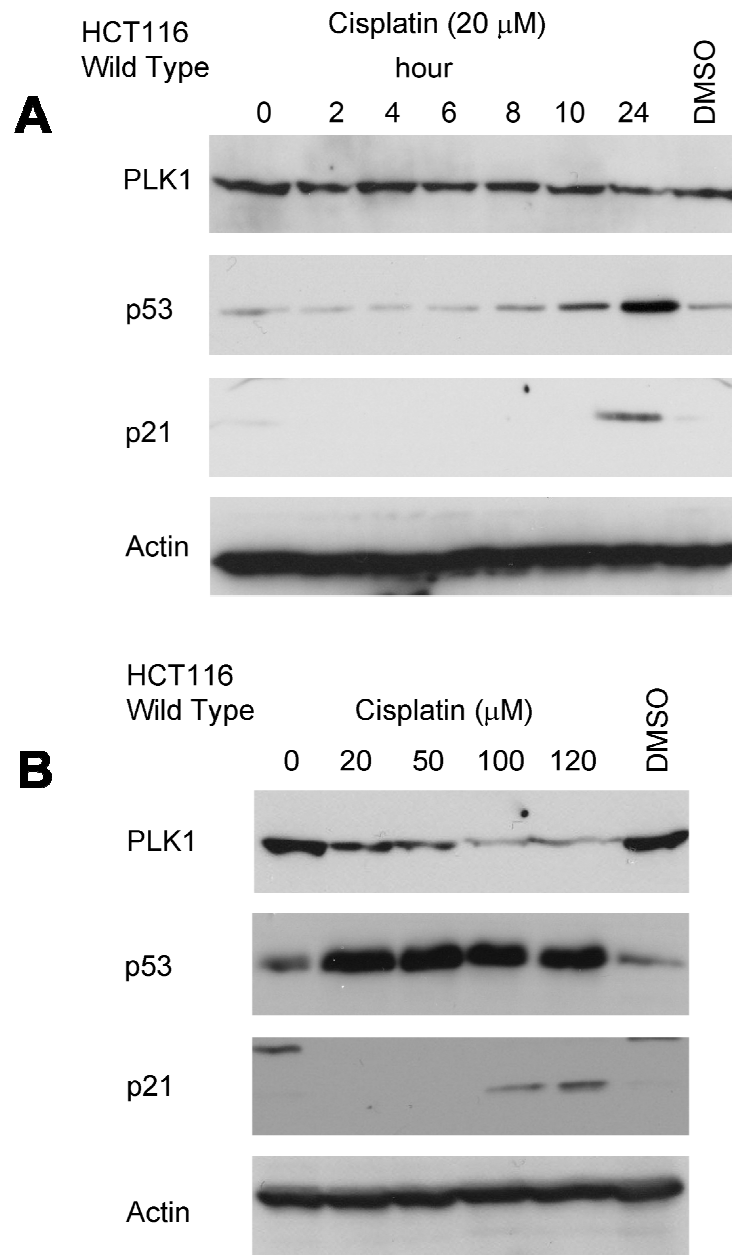


Figure 3.12: PLK1 down-regulation is concomitant with increasing levels of p53 after Cisplatin treatment in HCT116 wild type cells

A) HCT116 wild type cells were treated with 20 μ M Cisplatin and lysed at 2 h intervals over a 10 h period with a final time point lysed at 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments. **B)** HCT116 wild type cells were treated with increasing concentrations of Cisplatin for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 2 independent experiments.

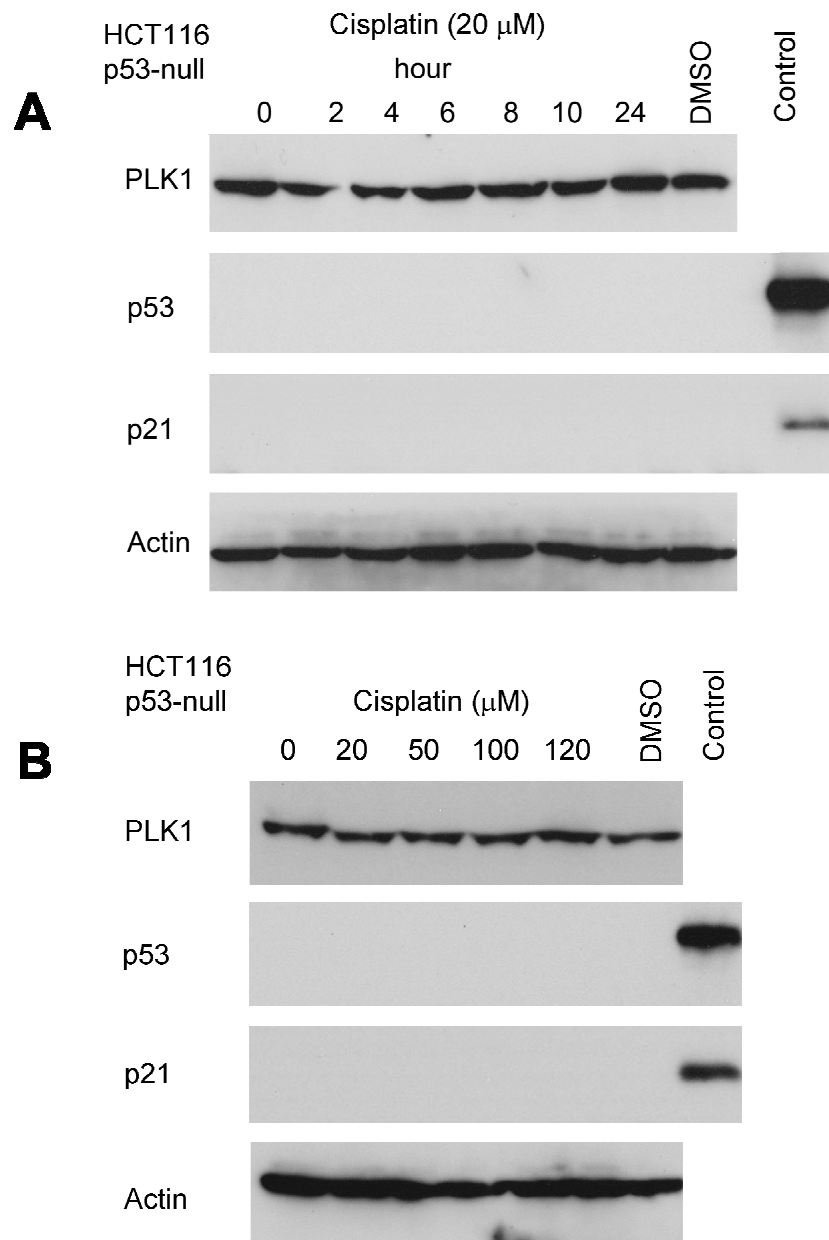


Figure 3.13: PLK1 down-regulation is concomitant with increasing levels of p53 after Cisplatin treatment in HCT116 p53-null cells

A) HCT116 p53-null cells were treated with 20 μ M Cisplatin and lysed at 2 h intervals over a 10 h period with a final time point lysed at 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments. **B)** HCT116 p53-null cells were treated with increasing concentrations of Cisplatin for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 2 independent experiments.

To determine whether p21 is involved in the down-regulation of PLK1 by Cisplatin, Cisplatin was also added to p21-null cells. As shown in Figure 3.14 (A), there was a small decrease in PLK1 which occurred concomitantly with a small increase in p53. Figure 3.14 (B) shows that with increasing concentrations of Cisplatin, levels of PLK1 decrease. Interestingly the levels of p53 decrease at higher concentrations of Cisplatin.

As with other stimuli, these experiments show that the down-regulation of PLK1 by Cisplatin is dependent on p53 and that this can occur in the absence of p21.

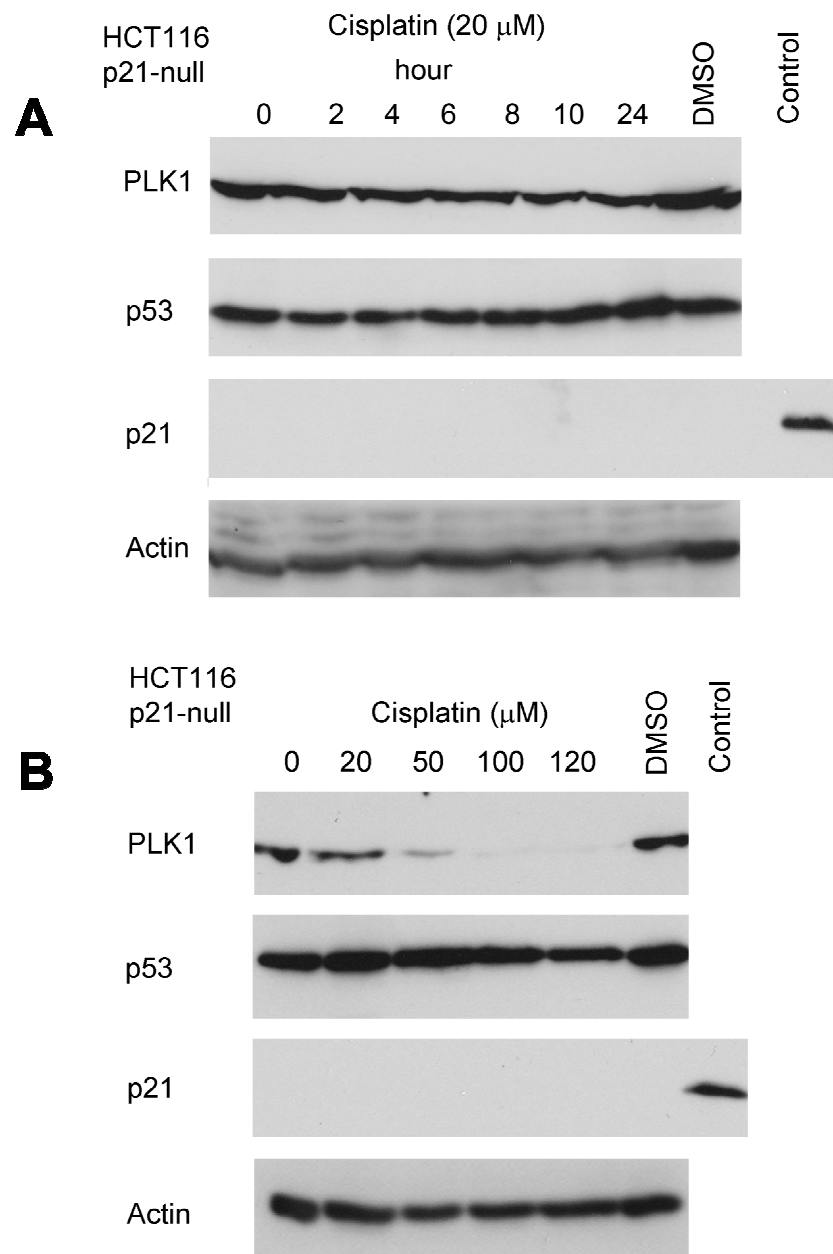


Figure 3.14: PLK1 down-regulation is concomitant with increasing levels of p53 after Cisplatin treatment in HCT116 p21-null cells

A) HCT116 p21-null cells were treated with 20 μ M Cisplatin and lysed at 2 h intervals over a 10 h period with a final time point lysed at 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 3 independent experiments. **B)** HCT116 p21-null cells were treated with increasing concentrations of Cisplatin for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies. These Western blots are representative of 2 independent experiments.

In conclusion, Etoposide, Nutlin-3 and Cisplatin all induce the expression of p53 in HCT116 wild type cells. This coincides with increases in MDM2 and p21 (Figure 3.20), and most notably, a decrease in PLK1. In the absence of p53 there is no decrease in PLK1 with any drug treatment. In the absence of p21 there is a small decrease in PLK1 levels when cells are treated with Nutlin-3. These results all point towards a possible repression of PLK1 by p53. It is likely that p21 makes a small contribution to this effect.

The requirement of p53, and p21, to down-regulate PLK1 is shown. It is also shown that both proteins are involved in checkpoint arrest. However, questions remain: how does p53 act to inhibit the cell cycle at the G2/M checkpoint? Is the down-regulation of PLK1 all that is required to cause G2/M checkpoint arrest? Since PLK1 plays a crucial role in the progression through late G2 and M towards cytokinesis, its down-regulation may mediate or contribute to the DNA damage induced G2/M checkpoint (Bunz *et al.*, 1998, Taylor *et al.*, 2001b). To explore this, PLK1 siRNA experiments were carried out.

3.10 p53 acts through a coordinated mechanism to repress PLK1

Results so far show that there are three drug treatments that result in the down-regulation of PLK1. To determine whether elimination of PLK1 would replicate or mimic the Nutlin-3 or Etoposide mediated G2/M checkpoint arrest observed in the flow cytometry experiments, PLK1 silencing (siRNA) experiments were carried out.

As shown in Figure 3.15, PLK1 levels were knocked down in the HCT116 cells and the p53- and p21-null derivatives. This experiment has included controls to account

for any affect the transfection reagents may have had and any unspecific affects the siRNA may have had. The knock-down in PLK1 is concomitant with increases in protein levels of the mitosis marker phospho-histone 3 (p-H3) and the apoptosis marker cleaved PARP (Poly(ADP) Ribose Polymerase) (Kaufmann *et al.*, 1993, Oliver *et al.*, 1999). In all three cell lines, interestingly, MDM2 levels decrease with decreases in PLK1, but there is no change in p53 protein levels. Furthermore, PLK1 levels were consistently reduced by a greater extent in the p53 and p21-null derivatives compared to the parental (p53-competent) cells.

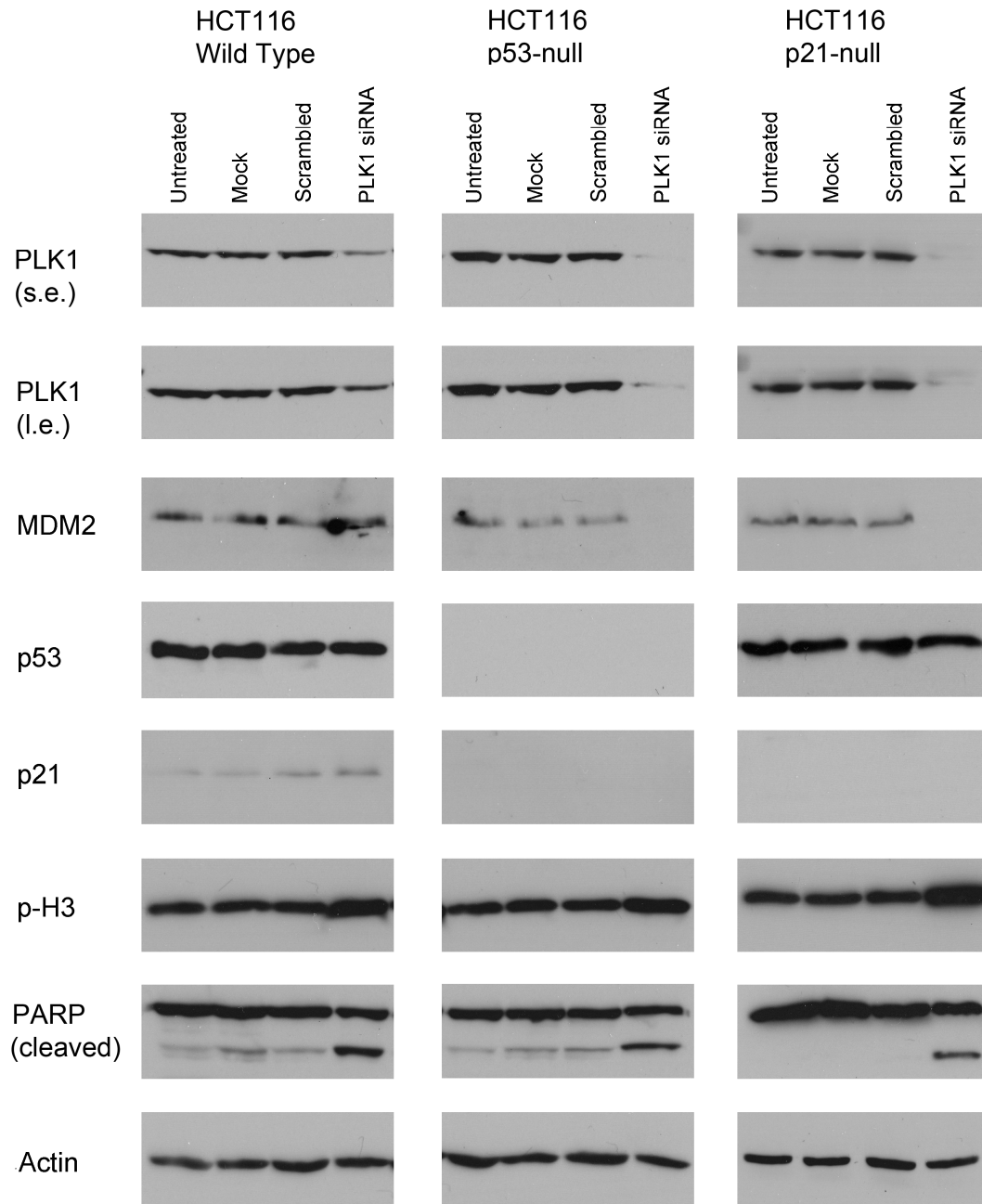


Figure 3.15: PLK1 knock-down by siRNA in HCT116 wild type, p53-null and p21-null cells

HCT116 wild type cells and their derivatives were treated with Optimem/Lipofectamine RNAiMAX only, Optimem/Lipofectamine RNAiMAX with Scrambled DNA (30 pMol) or Optimem/Lipofectamine RNAiMAX with *PLK1* siRNA (30 pMol). Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies, anti-phospho histone 3 antibodies, anti-PARP antibodies and anti-MDM2 antibodies. (s.e. = short exposure time, l.e. = long exposure time). These Western blots are representative of 4 independent experiments.

Western blots indicate that cells treated with PLK1 siRNA have entered mitosis and/or are committed to apoptosis. To confirm these suggestions, the cells were also examined by microscopy (Figure 3.16) and by flow cytometry (Figure 3.17).

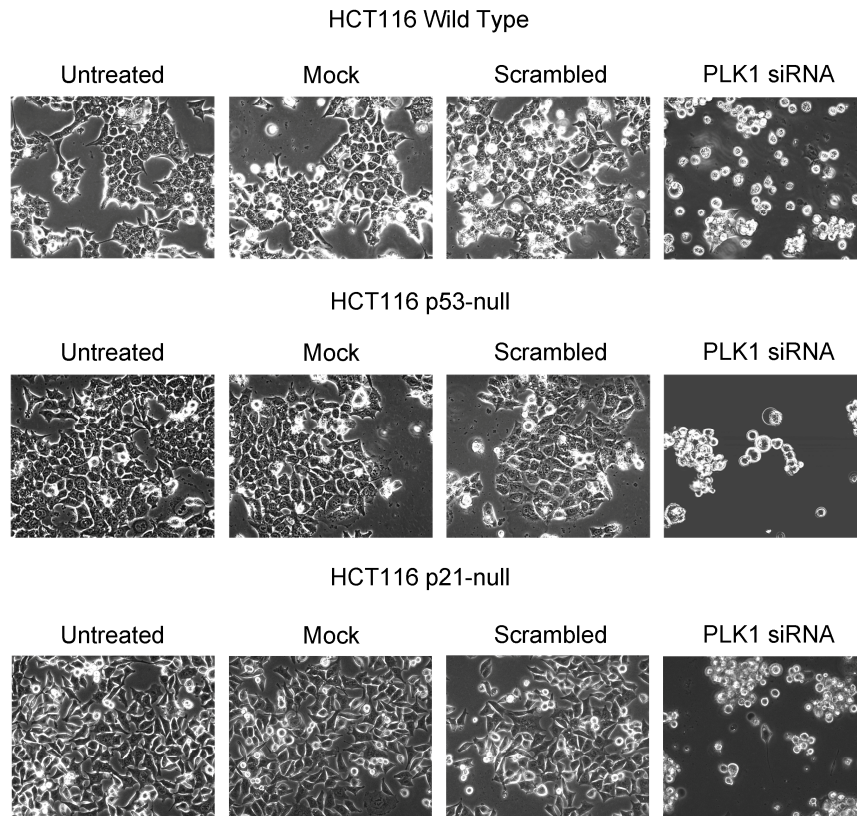


Figure 3.16: PLK1 knock-down by siRNA in HCT116 wild type, p53-null and p21-null cells

HCT116 wild type cells and their derivatives were treated with OptiMem/Lipofectamine RNAiMAX only, OptiMem/Lipofectamine RNAiMAX with Scrambled DNA (30p Mol) or OptiMem/Lipofectamine RNAiMAX with *PLK1* siRNA (30 pMol). Cells were then observed under the microscope and magnified to 200X, then photographed.

The micrographs in Figure 3.16 show that in all three cell lines, untreated cells, mock transfected cells and cells transfected with scrambled siRNA have a flat and normal morphology. In all three types of cell transfected with PLK1 siRNA, the cells' morphology was round and many were found floating in the medium, suggesting that the cells are either undergoing mitosis or going into apoptosis.

These micrographs support the observation with the Western blots. When PLK1 is knocked out, cells undergo mitosis and apoptosis. To confirm this, cells treated with *PLK1* siRNA were put through the flow cytometer (see Appendix). Figure 3.17 shows that the percentage of HCT116 wild type cells that are in G2/M phase and apoptosis increase when treated with *PLK1* siRNA. Figure 3.17 also shows that the proportion of p53-null cells and p21-null cells in G2/M phase increase when they are treated with *PLK1* siRNA. Similarly, there are increases in the proportion of sub-G1 cells consistent with the occurrence of apoptosis. The cell cycle profiles of cells either untreated, mock transfected or transfected with scrambled siRNA do not change, showing that the transfection itself is not having an effect on the result. Unfortunately, flow cytometry cannot discriminate between the cells in G2 phase and in mitosis. However, when the data from the Western blots is taken into consideration it can be concluded that the cells treated with PLK1 siRNA were in mitosis and not G2 phase.

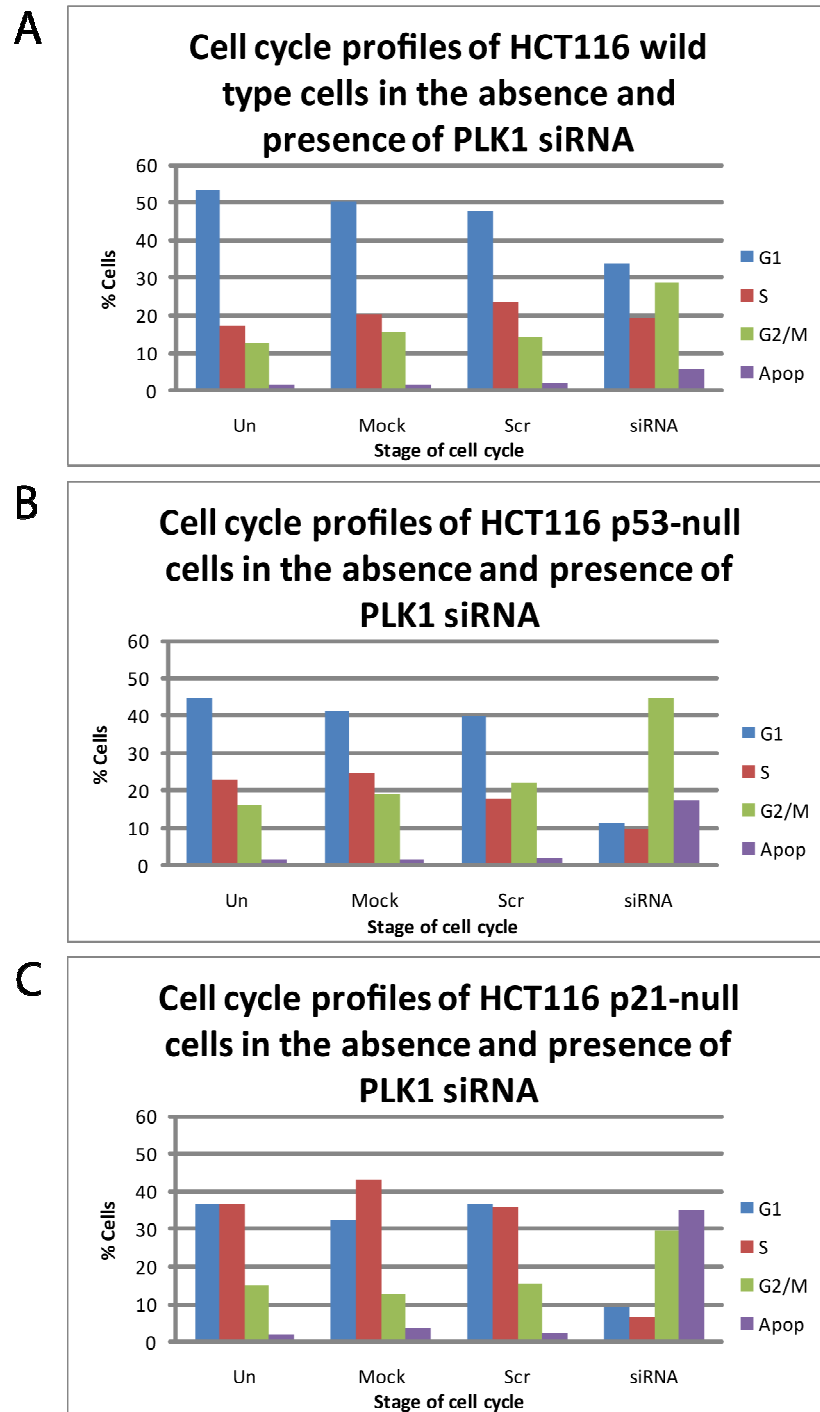


Figure 3.17: Cell cycle profiles of HCT116 wild type, p53-null and p21-null in the absence and presence of *PLK1* siRNA

This figure shows the flow cytometry data HCT116 wild type cells and their derivatives in the absence and presence of *PLK1* siRNA. Graph A shows the cell cycle profiles of the HCT116 wild type cells in the absence and presence of *PLK1* siRNA. Graph B, shows the HCT116 p53-null cells and Graph C, shows the HCT116 p21-null cells. These graphs are representative of 2 independent experiments.

The data presented here indicate that the knock-down of PLK1 in all three cell lines does not mimic the arrest mediated by Nutlin-3 and allows the cells to enter and arrest within mitosis. Since p53 arrests cells in G2 but not in M phase it is possible that down-regulation of PLK1 alone is not sufficient to prevent the cells from entering M phase and may rely on the coordinated action of p53 on this and several other genes.

Our next aim was to determine how p53 down-regulates PLK1 and whether this occurs at the transcriptional or post-translational level. Using the same drug treatments as previously described, Real-Time TaqMan[®] PCR was carried out and mRNA levels calculated, as described in Section 2.2.14.

3.11 p53 decreases levels of PLK1 mRNA levels

To determine whether down-regulation of PLK1 occurs at the mRNA level, HCT116 cells and their derivatives were treated with Etoposide or Nutlin-3, from which RNA was extracted and cDNA produced. From the cDNA, TaqMan[®] Real Time PCR was carried out to show if levels of expression of the *PLK1* gene, change in the absence and presence of Etoposide or Nutlin-3.

Figure 3.18 shows PLK1 mRNA levels decrease in HCT116 wild type cells when treated with Etoposide and Nutlin-3. PLK1 levels do not change in the HCT116 p53-null cells when treated with Nutlin-3 and increase slightly when treated with Etoposide. In p21-null cells treated with Etoposide PLK1 mRNA levels increase. It is not clear whether there is decrease in PLK1 mRNA levels mediated by Nutlin-3.

Power analysis revealed that the study would have to be replicated at least 6 times for such a small effect to be statistically significant.

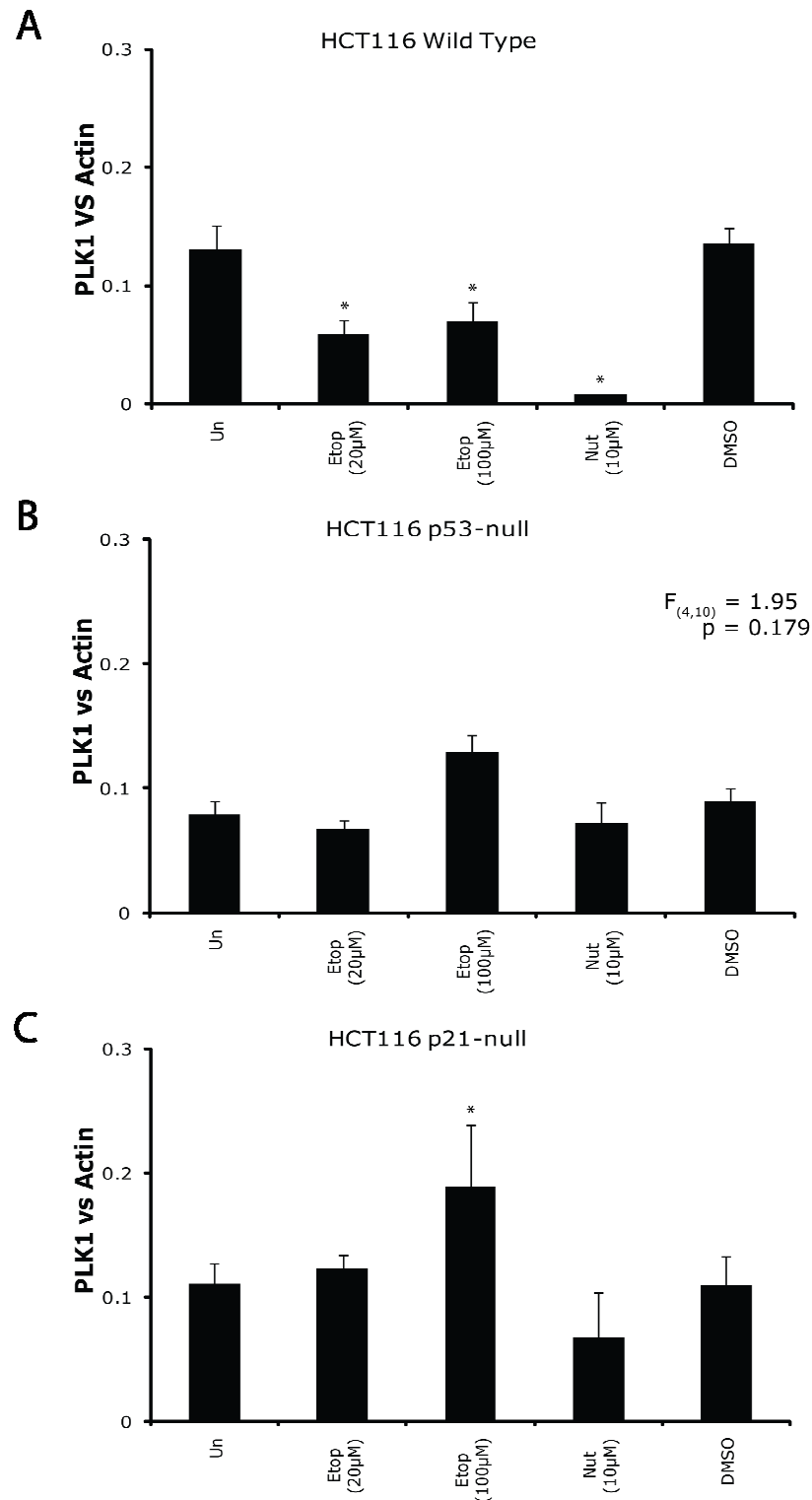


Figure 3.18: Real-Time TaqMan® PCR data of the Etoposide and Nutlin-3 treated HCT116 Wild Type, p53-null and p21-null cells

HCT116 wild type (A), p53-null (B) and p21-null (C) cells were treated with Etoposide (20 µM, 100 µM), Nutlin-3 (10 µM) or DMSO for 24 h. RNA was extracted from the lysed cells (as described in Chapter 2) and cDNA produced from the RNA. Real-Time TaqMan® PCR was then performed and levels of mRNA calculated. These graphs are representative of 3 independent experiments. Statistical analysis was performed using analysis of variance with post hoc Bonferroni test for multiple comparisons (* $p < 0.05$).

In conclusion, TaqMan[®] Real-Time PCR shows that levels of PLK1 mRNA levels parallel the changes observed at the protein level. Analysis of variance revealed that in the presence of p53, PLK1 levels decrease when HCT116 wild type cells are treated with Etoposide and Nutlin-3. In the absence of p53 there is no decrease suggesting that PLK1 repression at the mRNA level is dependent on p53. PLK1 mRNA levels in p21-null cells increase in the presence of Etoposide. Time permitting other time points would also have been followed up, to discover whether mRNA levels differed at other time points compared to the protein levels.

Our next aim was to elucidate the mechanism by which p53 mediates PLK1 down-regulation and whether any other proteins may be involved in these events. One plausible mechanism, suggested by the data so far, is transcriptional repression. This can involve either binding site overlap or even squelching of transcriptional activators but very commonly involves the recruitment of chromatin remodelling proteins, such as histone deacetylases (HDACs), therefore the next Section explores whether HDACs are involved in PLK1 repression via p53.

3.12 Histone deacetylases may be involved in repression of PLK1

In order to determine whether p53 down-regulates PLK1 mRNA levels through a repression mechanism involving histone deacetylases, an experiment was carried out using the HDAC inhibitor, Trichostatin A (TSA). As shown in Figure 3.19, HCT116 cells (parental and p53-null) were treated with either: TSA, Nutlin-3, both TSA and Nutlin-3 or DMSO. The data indicate that TSA alone inhibits the expression of PLK1. Nutlin-3, also shown previously, represses PLK1 to very low levels. Interestingly however, when cells were treated with both drugs, TSA rescued the

PLK1 levels to the levels of TSA alone. Western blotting of acetyl-histone 3 (acetyl-H3) shows that the TSA drug itself is active and has caused an increase in acetylated H3 as expected. Western blotting confirmed that Nutlin-3 stimulated an increase in p53, p21 and MDM2.

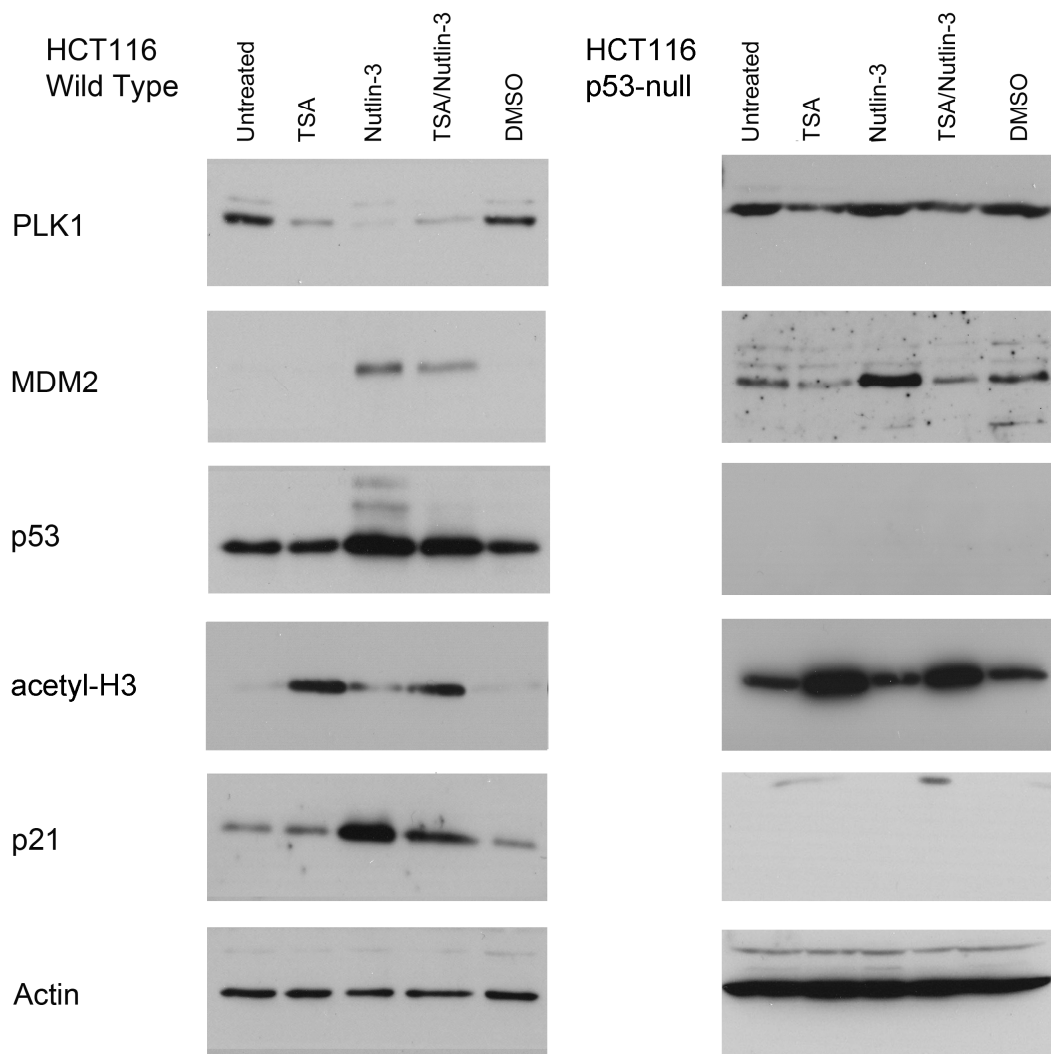


Figure 3.19: Trichostatin A (TSA) causes a decrease in the levels of PLK1 protein independent of p53 but alleviates p53 mediated PLK1 down-regulation

HCT116 wild type and p53-null derivatives were treated with Nutlin-3 (10 μ M), TSA (1 μ M) or both for 24 h. Lysates were then analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-p21 antibodies, anti- β -Actin antibodies, anti-acetyl-H3 antibodies and anti-MDM2 antibodies. These Western blots are representative of 4 independent experiments.

These data fit with the idea that p53 may repress the expression of PLK1 through a mechanism involving the recruitment of HDAC to the PLK1 promoter.

3.13 Concluding Remarks

Down-regulation or repression by p53 is an exciting area of research that has previously received less attention than analysis of the activation of gene expression by p53. This chapter shows and discusses the down-regulation of a particular protein (or oncogene), PLK1, by the transcription factor and tumour suppressor, p53. PLK1 is a cell cycle regulated protein and required for progression through G2 phase and mitosis (Hamanaka *et al.*, 1995, Petronczki *et al.*, 2008). It is also a protein that is deregulated and/or overexpressed in many types of cancer (Holtrich *et al.*, 1994, Kneisel *et al.*, 2002).

Data in this chapter show that treating particular cell lines, such as HCT116 with drugs such as Etoposide and Nutlin-3 causes PLK1 levels to decrease. However levels do not decrease in the absence of p53. These conclusions are exemplified in the experiment as shown in Figure 3.20. Increases in p53 occur with increases in MDM2, p21, 14-3-3 (three well-characterised targets of p53) (Ko and Prives, 1996). To eliminate the possibility that the observed decrease in PLK1 levels was simply the result of p21 induced G1/S checkpoint arrest (i.e. arresting the cells at a point where the levels of PLK1 are normally low) flow cytometry was carried out. This data confirmed that down-regulation of PLK1 did not occur as a result of the inhibition of the cells cycle when PLK1 levels are low. This down-regulation has been further confirmed by data from our lab (Dr Sharon King) which suggest that induction of p53 in SAOS2 cells that have inducible p53, down-regulates PLK1;

(SAOS2 cells do not have Rb and therefore do not arrest at G1/S (Qin *et al.*, 1995, Weinberg, 1995)).

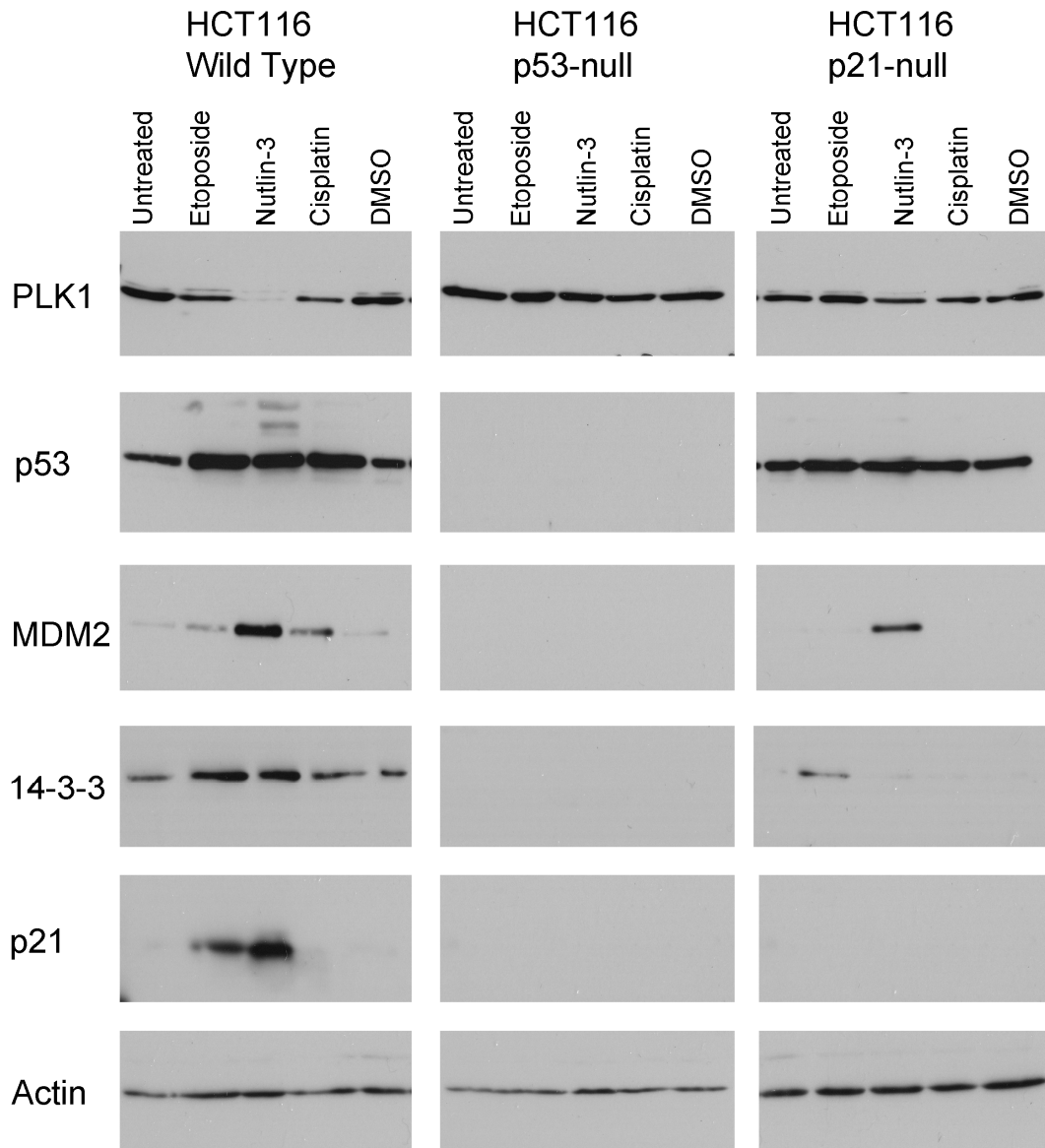


Figure 3.20: PLK1 down-regulation is dependent on p53 and, in part, p21

HCT116 wild type, p53-null and p21-null cells were treated with Etoposide (100 μ M), Nutlin-3 (10 μ M) or Cisplatin (20 μ M) for 24 h. Cells were then lysed and analysed by Western blotting with anti-PLK1 antibodies, anti-p53 antibodies, anti-MDM2 antibodies, anti-14-3-3 antibodies, anti-p21 antibodies and anti- β -Actin antibodies.

p21 may have a growth arrest independent role to play in down-regulation of PLK1. For example, down-regulation of PLK1 by Nutlin-3 still occurs in p21-null cells but is less potent than in the parental cell line. Similarly, silencing of p21 expression in *p21* siRNA experiments indicated that p21 makes a contribution to the down-regulation of PLK1.

Flow cytometry experiments were carried out to determine whether these drugs were affecting the cell cycle profile of the cells and inhibiting the cell cycle at the point when PLK1 levels are naturally low. The data confirmed that cells do not arrest at the G1/S checkpoint when p53 or p21 are absent. Interestingly, DNA damaging reagent Etoposide caused a G2 block that was not dependant on either p53 or p21. It is likely, in this case, that Etoposide being a DNA damaging reagent, is activating the ATM/CHK2 pathway leading to a p53-independent G2 cell cycle arrest (Bartek and Lukas, 2003, Eymin *et al.*, 2003). Nutlin-3, on the other hand, displayed a G1 block and G2 block, which was noticeably dependent on p53 and p21 as it occurred only in the parental cells (Nutlin-3 activates the p53 pathway by inhibiting the interaction of p53 with MDM2). Taken together, the data indicate that activating p53 is sufficient to down-regulate PLK1, independently of its ability to arrest cells at G1/S.

Interestingly, the knock-down of PLK1 does not cause G2/M checkpoint arrest and cells become blocked in mitosis and undergo apoptosis. Therefore, this suggests that inhibition of PLK1 alone is not sufficient to mediate the G2/M checkpoint and that p53 may act in a coordinated mechanism in which it requires coordinate regulation of additional genes to mediate the G2/M checkpoint block. Alternatively, since G2/M arrest can occur independently of p53 (at least transiently), down-regulation of

PLK1 may serve to enforce the arrest by eliminating one of the key factors required for passage through mitosis. This would fit with the idea that the function of p53 lies in its ability to maintain the G2/M checkpoint as opposed to activating it.

To determine whether PLK1 down-regulation occurs at the transcriptional level, PLK1 mRNA levels were measured. The data indicate that down-regulation occurs at mRNA level. Therefore, our aim was to determine how p53 acts to down-regulate PLK1 and what other factors this may involve.

The down-regulation of PLK1 at the mRNA level suggested that p53 might repress PLK1 expression. Repression often occurs through the recruitment of proteins such as HDACs that remodel chromatin such that it is less accessible to the transcriptional apparatus. HDACs are well-established inhibitors of gene expression and so a further experiment was carried out to determine whether the mechanism of p53 repression of PLK1 involves the recruitment of HDACs. The initial data showing that the HDAC inhibitor TSA can relieve the down-regulation of PLK1 in response to Nutlin-3 treatments fits with the suggestion that HDACs are involved in repression of PLK1.

Overall the results in this chapter give evidence of a repression mechanism of PLK1 that is dependent on p53 and partly on p21. The next chapter explores the mechanism by which p53 and perhaps p21 carry out repression of PLK1 and provides a model by which this may occur.

Chapter 4: p53 repression of PLK1

4.1 Background

PLK1 is down-regulated by p53 however the mechanism by which it does this is unclear. p53 may act directly through proposed p53 responsive elements (p53 REs), like that of Survivin (Hoffman *et al.*, 2002, Riley *et al.*, 2008) or indirectly such as the activation of p21, which may have an effect through other regions of the *PLK1* promoter (Shiyanov *et al.*, 1996, Uchiumi *et al.*, 1997). It is also possible that p53 can interfere with important factors such as NF-Y (Imbriano *et al.*, 2005).

This Chapter describes the use of several complementary approaches to determine whether p53 mediates transcriptional repression of PLK1. Firstly, the *PLK1* promoter was cloned into a Luciferase reporter vector, which was then transfected into HCT116 p53-null and SAOS2 cells. Luciferase assays were then carried out on the lysates to determine whether p53 causes a decrease in Luciferase levels (i.e. as a read-out of p53 transcriptional function). Other techniques were also employed, such as Chromatin immunoprecipitations, to establish whether p53 acts directly on the *PLK1* promoter and, if so, where this occurs. Furthermore, an electrophoretic-mobility shift assay was also carried out to confirm, *in vitro*, the binding of p53 to a proposed p53 RE.

Lastly this Chapter explores whether p21 has a role to play at the promoter level in the repression of PLK1.

4.2 Aims

The aim of this Chapter was to explore the mechanism(s) by which p53 represses PLK1.

4.3 Cloning of the *PLK1* promoter into a Luciferase reporter vector

As suggested in the previous Chapter, PLK1 is down-regulated by p53 and, in part, p21. The *PLK1* promoter contains many potential targets for site-specific transcription factor binding including sites including an E2F binding site, CDE (cell cycle-dependent element)/CHR (cell cycle gene homology domain) repressor element and CCAAT box, as shown in Figure 4.1. The promoter sequence of human *PLK1* (accession number X90725) was also screened for potential p53 binding sites using software developed at Dundee to detect p53 binding sites (by Ms Sieu Chin, Dr J-C Bourdon's group, non-commercial). The algorithms employed by this computer programme detect the consensus sequence of a p53 responsive element which is made up of the following: PuPuPuCA/TT/AGPyPyPy. The computer programme detects any potential site that contains two to three purines (G/C) at the start of the sequence followed by a C and either an A or T. The C is not interchangeable. The programme also detects sites that contain two to three pyrimidines (A/T) followed by a G and either an A or C. The G is not interchangeable. The final algorithm employed takes into account any spacer that occurs in p53 responsive element. This is usually 0-13 bp long.

This approach identified three potential p53 binding sites, two of which were overlapping (p53RE1) as shown in Figure 4.1. The sequence of *PLK1* promoter which was cloned into the Luciferase reporter vector is shown between the yellow highlighted primers (Figure 4.1)

p53 may act directly at the p53 REs to repress PLK1. On the other hand, based on published analyses of other promoters (St Clair *et al.*, 2004) the induction of p21, by p53, may repress PLK1 in conjunction with or independently of p53. p21 may

repress PLK1 through the CDE/CHR element (Lucibello *et al.*, 1995, Uchiumi *et al.*, 1997) and/or through the E2F binding site (Shiyanov *et al.*, 1996). There are also additional sites, through which repression may occur (discussed in Chapter 1). In this Chapter we explore repression of PLK1 by p53 through the p53 REs and investigate whether this may involve p21 repression of PLK1 through the CDE/CHR element. Future study will reveal whether repression occurs through other domains.

PLK1 promoter sequence

```

1581 aacaaaaagg aattaaggag gaagccacct ggcttcctaa
1621 gatagacttt atggttaaat ggatttatTT tagctgggta agtccaggac ccctaccagg
1681 aagaactgag gtccatattt gaggttggtc tcacccttTT gcatgtgggc ctactaataa
1741 taattttgct gtaaagaagc ctcaataagg agatggactg tgtgaaaaga gatgagaaag
1801 caggcctggt tgcaaggcgt gcccttggtc ggcatctgaa agcttgattt tcaggagggt
1861 totcattact ccctaaatga taagagtggc tcaactgtgc taaactgttc gtacaaacaa
1921 tgtgggttat gcccaacatc tgccttcctt ctgggagtca gattttcata tgtgttaggc
1981 agaggatgag gtgagcagct cccaatgaga accctgaaca ctgagctctgt aatgagcttc
2041 ccttgtatag aacattgcac atgggttggt acaactgatt gctggaggaa ttgtgtccta
2101 tgtgactctg ctgggagagg actgtgggag gcttacacct ggtttccttg gactttgtcc
2161 atgcgctttt ttcttttgcg gattttgctt cctagccttt cgtgtagta aaacatagcc
2221 atgagtatga ctacaggctg agtctgtgaa tctcctagta catcatcaga ctaggagggtg
2281 gtgggtgttg cccccccag cacagggcac aagggagact tgcagggtgt ttgtcatgtt
2341 cccttttcca atctgggtgt gggttacaga tttgttcagt ttgtgaaat tcactgagct
2401 ctatgaacaa ttataatatg tacatttttt tctgtaagtg tattaatttt caataaaaag
2461 aattcacact ataggggtac aaaataactt aatttttcaa agtcttcctt ttattcagcc
2521 aacatttttag taagtaccct ttttttttta ccaagcactg ctctgggagc ttgggacatg
2581 gggtagaaag gacggcatca ttgtcctcat tgtccttcag cttagctgca aggcagggtga
2641 aggatgctta tagtatggtt gatttttgct gggtctctat aaggatcaga ggggtctaag
2701 gaggcacaga gaaagagctt ttaacgtggg tattttactg ttccagggtt gccactcaag
2761 tacctacaga acaaacgggt gtagctgtaa actgtggcaa actgaaagca tgtaggcaca
2821 gagtaagtag ctgagctgta tttttttcag gaaaggccac aaatcccaac ttttatgagc
2881 toctctttta aatgctgtga atgttttaca atggcggggc gcggtggctc acgctgttaa
2941 toccagcatt tgggaggccg aggggcagat cacttgagggt caggagtcca agaccagcct
3001 ggccaacatg gtgaaacctc ttctctaact aaattacaaa aattagccgg gcatgggtgt
3061 gcatgcttgt aattccagct gctaggaggg ccgaggcggg aggattgctt gaacccggga
3121 agcagaggtt gcagtgaact gagatcgtgc cactgcactc cagcctgggc gacagagcaa
3181 gattccgtca cacacacaaa aaaaaggcgt ggggggaggc caaacaaaac cccgcaagac
3241 acatttggtt atgacctgcc agtttgctag gcatctctcc aaccttcctt ccctctgacc
3301 aagaaactga gtgtccacta ttttaggccc tgggaaattc agtagcgagg aggccagaca
3361 gcttcgttgc atcatggggg gctctgttac tgtgcttctc caacttcagg atgtgtagga
3421 atcacctgag cagtcttggt gagaggcgga cactgactcg ggaggtctgg ggttagggct
3481 gaacgtttgc ctttgoggtt ctaacaagct ctcagggtgat ggcgatgcta ctgttccctg
3541 gcccgagggt agaggaagat ttaagtataa gcttcctgga ggaggcgcaa gtgaaccgca
3601 ggagctttcc cggacgcccg agaaggggaga aaccccgagg gaattcctcc tctctggggg
3661 ctgggtctcc gcatccacgc cgggtttggt ttcccaggct atcccacgtg ttccggcgctc
3721 cgtgtcaatc aggttttccc cggctgggtc cgggtttaa ggctgctgct gcgcagggcg
3781 ctcccatggt gccgcggggc gggcggtttt ggatttttaa tccccgcggc caatcagtggt
3841 cgcgcagggt tttgtaacgt tcccagcgdc gcgtttgaat tcggggaggga gcggagcggt
3901 tgcggaggct ctgctcggat cgaggctctg agcgcagctt cgggagcatg

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Elements/Sites in <i>PLK1</i> promoter	Start of Sequence	End of Sequence
PCR primers	1581, 3873	1601, 3893
p53 responsive element 1	1719	1849
p53 responsive element 2	3001	3080
E2F binding site	3735	3743
ccat box	3830	3834
CDE/CHR repressor element	3866, 3875	3869, 3879
Transcriptional and translational start sites	3865, 3948	-

Figure 4.1: Diagram of *PLK1* promoter showing proposed sites, determined sites and other elements

Diagram of *PLK1* promoter depicting the proposed p53 responsive elements (red) and E2F binding site (blue), ccaat box (pink), CDE/CHR repressor elements (purple), transcriptional and translational start sites (green) and the PCR primers (yellow) used to amplify the *PLK1* promoter. Arrows indicate base pairs that correspond to the p53 consensus sequence.

The *PLK1* promoter was cloned into a Luciferase reporter vector (pGL3) as shown in Figure 4.2.

Cloning of the *PLK1* promoter into the Luciferase reporter vector, pGL3, via the subcloning of *PLK1* promoter into the cloning vector, pSC-B

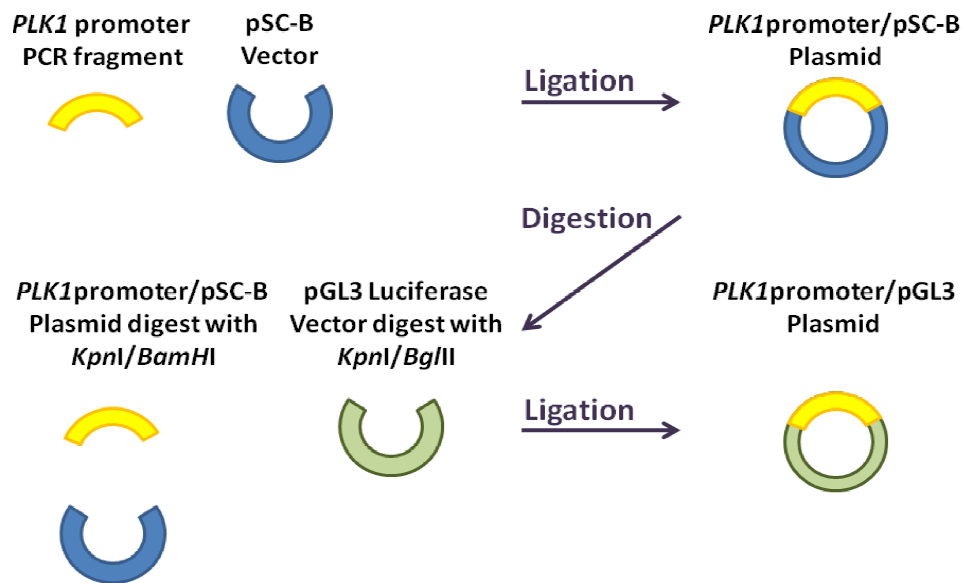


Figure 4.2: Cloning of the *PLK1* promoter into the Luciferase reporter vector, pGL3, via the pSC-B vector

The cloning of the *PLK1* promoter involved the amplification of the promoter by PCR which was then ligated into the vector, pSC-B. This was then excised by restriction digest into two fragments. The fragment containing the *PLK1* promoter was then ligated into pGL3.

The pGL3 vector, as shown in Chapter 2, has a cloning site which is directly upstream of a *Luciferase* gene and therefore the cloning of the *PLK1* promoter into this vector allows transcription of the *Luciferase* gene and expression of Luciferase. Consequently, the levels of Luciferase are a reflection or read-out of the activation of transcription and translation of the *PLK1* promoter. The cloning of the promoter into

this particular vector allows us to measure the effects of ectopically expressed wild type and mutant p53 proteins on expression from the *PLK1* promoter. Moreover, if down-regulation of expression requires an element(s) within this promoter it would underpin the idea that p53 represses PLK1 expression.

To create the *PLK1*promoter/pGL3 plasmid, the *PLK1* promoter DNA was amplified from a BAC clone, as described in Chapter 2, using PCR (Figure 4.3). The PCR product was then excised from the agarose gel and purified before being ligated into the pSC-B plasmid vector from the StrataClone™ Blunt PCR Cloning Kit, as described in Chapter 2. The *PLK1* promoter is ligated into the pSC-B plasmid vector prior to ligation in to pGL-3 plasmid as PCR products can be directly ligated into the pSC-B vector.

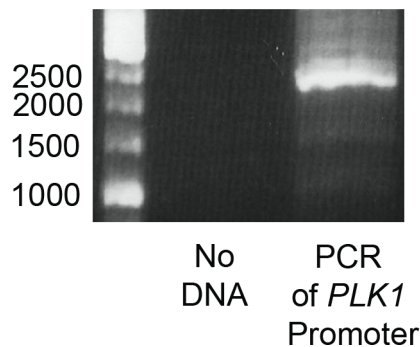


Figure 4.3: PCR amplification of the *PLK1* promoter

PLK1 promoter DNA was amplified from a BAC clone as described in Chapter 2. A PCR reaction containing no DNA was also performed and run on the gel to show there is no DNA contamination.

After ligation, the plasmid was then used to transform StrataClone SoloPack competent bacteria to Ampicillin resistance. Plasmid DNA was then purified from the bacteria for further use. To verify that the fragment containing the *PLK1* promoter was inserted correctly into the vector and the ligation was successful, a double restriction digest was carried out. Using restriction enzymes, *XhoI* and *BamHI*, the plasmid was digested and two fragments of size, 2384 and 3388 Kb

(which were of the size expected from this digest) were observed as shown in Figure 4.4. These enzymes were chosen as the plasmid contains both these restriction sites and the digest would give two DNA products of these predicted sizes.

*PLK1*promoter/pSC-B Digest

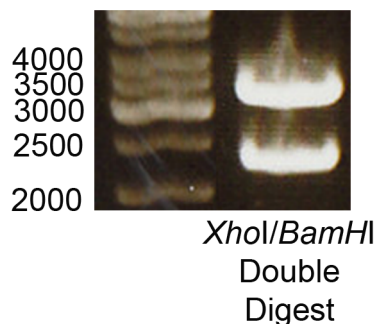


Figure 4.4: Double restriction enzyme digestion of *PLK1*promoter/pSC-B plasmid

The *PLK1*promoter/pSC-B plasmid was digested using the restriction enzymes *XhoI* and *BamHI*.

To clone the *PLK1* promoter into the pGL3 Luciferase reporter vector further double restriction enzyme digests were carried out. Using *KpnI* and *BamHI* restriction enzymes, the *PLK1* promoter was removed from the pSC-B vector (Figure 4.5). These two enzymes were chosen because the insert containing the *PLK1* promoter is flanked by *KpnI* and *BamHI* restriction sites and are complementary sites to the vector flanked with sites *KpnI* and *BglIII*. Using *KpnI* and *BglIII* restriction enzymes, the pGL3 vector was digested into a linear form of the plasmid (Figure 4.5). After digestion, the *PLK1* promoter, size 2.3 Kb and pGL-3, size 4.8 Kb were excised from the agarose gel and purified. The *PLK1* promoter was then ligated into the pGL3 Luciferase reporter vector, as described in Chapter 2.

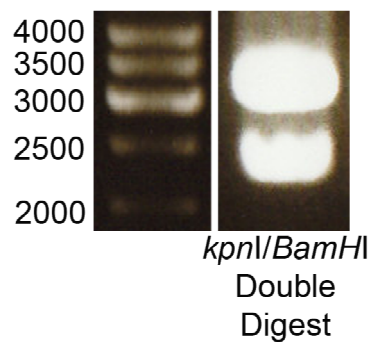
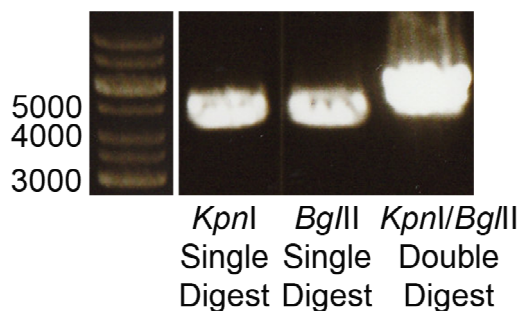
PLK1 Promoter Digest out of pSC-B

Figure 4.5: Double restriction enzyme digestions of *PLK1* promoter/pSC-B plasmid and pGL3 vector

PLK1 promoter/pSC-B plasmid was digested using the restriction enzymes *KpnI* and *BamHI*, which create two bands of size 2.3 and 3.5 Kb. The pGL3 vector was digested using the restriction enzymes *KpnI* and *BglII*, giving a band of size, 4.8 Kb.

pGL3 vector Digest

Subsequent to ligation, the plasmid was then used to transform competent *E.coli* DH5 α to Ampicillin resistance. Plasmid DNA was then purified from the bacteria for further use. To show that the fragment containing the *PLK1* promoter was inserted into the correct vector and the ligation was successful, a double restriction digest was carried out. Using restriction enzymes, *XbaI* and *KpnI*, the plasmid was digested and two fragments of size, 4045 and 3062 Kb were observed (which were of the size expected from this digest) as shown in Figure 4.6. These enzymes were chosen as the plasmid contains both these restriction sites and the digest would give two DNA products of a predicted size.

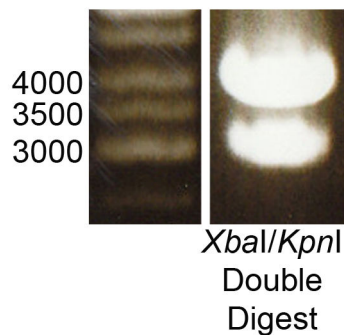
*PLK1*promoter/pGL3 Digest

Figure 4.6: Double restriction enzyme digestion of *PLK1*promoter/pGL3 plasmid

*PLK1*promoter/pGL3 plasmid was digested using the restriction enzymes *KpnI* and *XbaI*.

Furthermore, sequencing (DNA analysis Facility, University of Dundee) was carried out to verify that the promoter had been correctly inserted into the plasmid and that no undesired mutations had arisen.

The construction of this plasmid has allowed us to determine whether p53 represses *PLK1* at the promoter and is described in the next Section.

4.4 p53 represses expression from the *PLK1* promoter

To determine whether p53 mediates *PLK1* repression, the *PLK1*promoter/pGL3 plasmid was initially transfected into H1299 cells (which are p53-null). Results showed that the pGL3 vector alone was subject to express Luciferase in increasing amounts of p53. This indicated that p53 alone was causing Luciferase expression from an area in the pGL3 vector. Therefore HCT116 p53-null cells were used, and the plasmid was transfected into HCT116 p53-null cells together with increasing amounts of p53 expression plasmid, and a plasmid encoding *Renilla* Luciferase as internal standard (described in Chapter 2).

After 24 h, cells were lysed and Luciferase assays carried out, as described in Chapter 2. Levels of Luciferase are normalised to levels of Renilla. As shown in Figure 4.7 (A), as wild type p53 levels increase the expression of Luciferase decreased. At 300 ng of p53 plasmid, the decrease in levels of expression of Luciferase, had reached a plateau, which was reached long before this concentration. To confirm that the reduction in Luciferase levels was dependent on wild type p53 function, various forms of mutant p53 were substituted for the wild type p53. These included: the R175H mutant, which is unable to bind to DNA because of a conformational change in the site-specific DNA binding domain. The R273H mutant, which has a mutation at the DNA binding domain which does not cause a conformational change but which eliminates a residue important for DNA contact and the L22Q/W23 mutant, which has two mutations in the TAD1 (trans-activating domain) region of p53 that binds to the co-activator, p300 (corresponding p53 residues 25 and 26, in murine cells, are required for p53 dependent activation and repression (Hammond *et al.*, 2006)). As shown in Figure 4.7, neither the R175H nor the R273H mutants repress the expression of Luciferase. Since neither of these mutants has DNA binding ability it is likely that site-specific DNA binding is required for p53 to mediate PLK1 repression. This Figure also shows that the L22Q/W23S mutant of p53, which cannot trans-activate gene expression, is also unable to repress expression of Luciferase from *PLK1* promoter.

In conclusion these results show that only wild type p53 can repress PLK1 expression through the *PLK1* promoter.

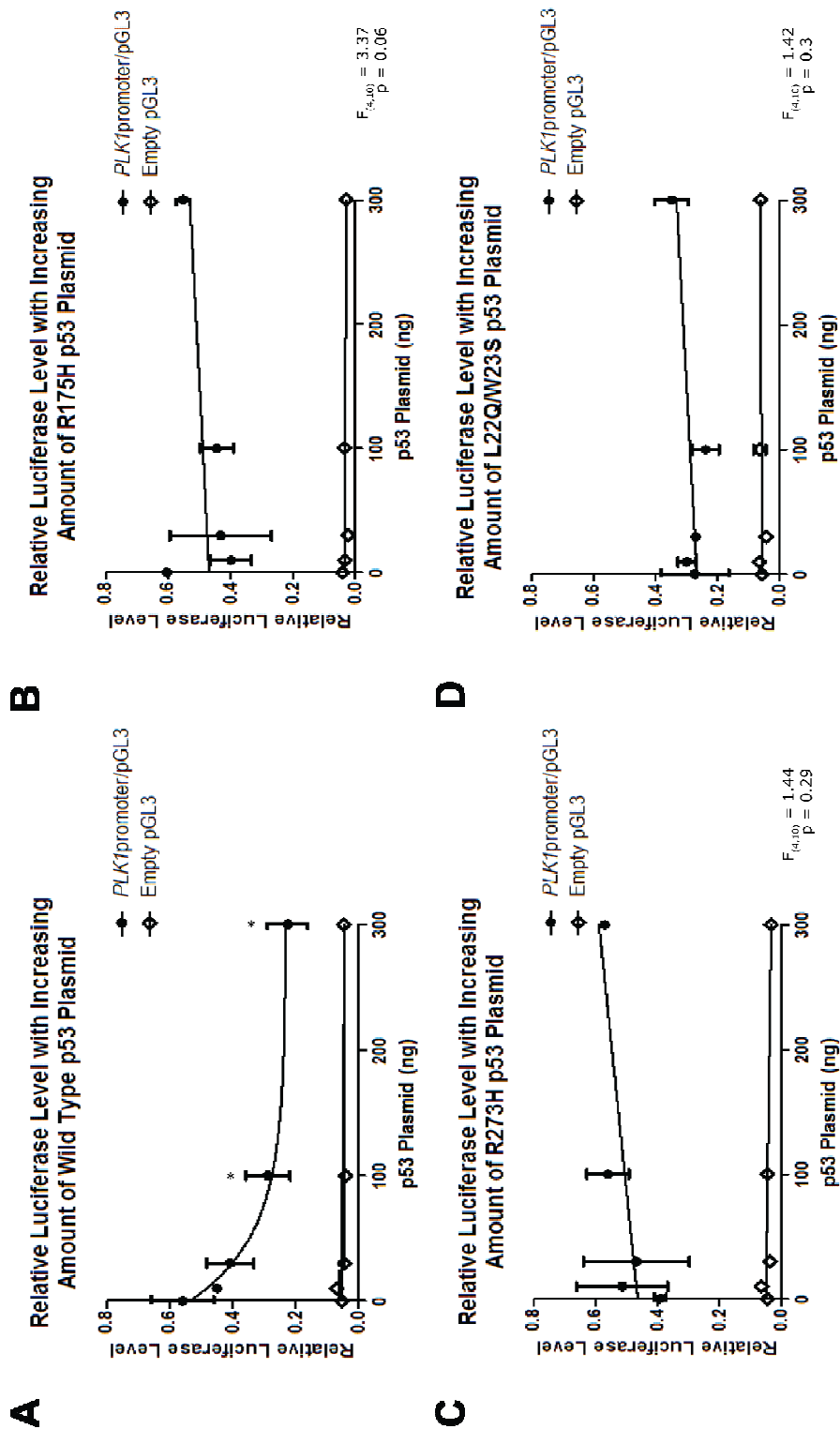


Figure 4.7: Relative Luciferase levels in the presence of increasing amounts of p53 and p53 mutants R175H, p53 R273H and p53 L22Q/W23S in HCT116 p53 null cells.

p53 plasmids were transfected together with *Renilla* plasmid, *PLK1*/promoter/pGL3 plasmid or empty pGL3 plasmid and empty PCDNA3 plasmid into HCT116 p53-null cells. After 24 h cells were lysed and a Luciferase assay carried out on the lysates. Luciferase activity levels were then read by a luminometer. Variations in transfection efficiencies were corrected by determining the ratio of activity of Luciferase to *Renilla*. These graphs are representative of 5 independent experiments. Statistical analysis was performed using analysis of variance with post hoc Bonferroni test for multiple comparisons (* $p < 0.05$).

To demonstrate that the p53 plasmid had been transfected into cells and levels of p53 increased with increasing amounts of p53 plasmid, Western blots were performed (Figure 4.8). This Figure confirms that p53 levels increase dose-dependently. However, it was not possible to detect p21 levels under these conditions.

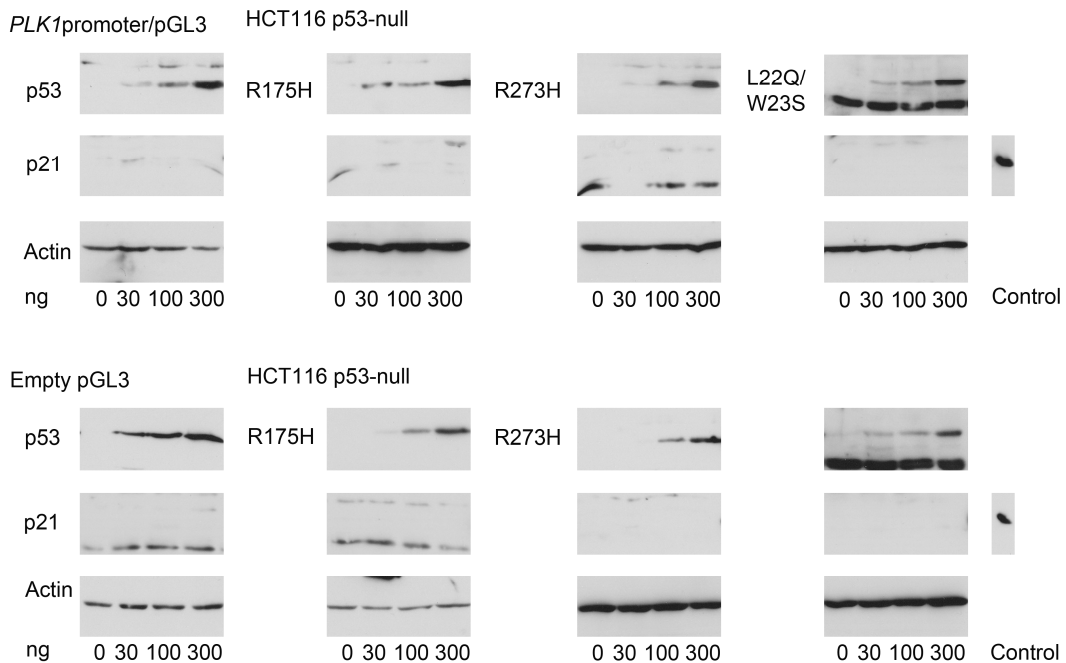


Figure 4.8: p53 levels increase in a dose-dependent manner

HCT116 p53-null cells were transfected with either wild type p53 plasmid or mutant p53 plasmids R175H, R273H or L22Q/W23S together with *PLK1*promoter/pGL3 plasmid or empty pGL3, *Renilla* plasmid and empty PCDNA3 plasmid. Lysates were then analysed by Western blotting with anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies.

Our collaborator had shown by ChIP that p53 interacts with the *PLK1* promoter in proximity to p53RE2 but had not been able to detect p53 either at p53RE1 or at the core promoter (Sam Nicol, unpublished data). While I was unable to follow up on these ChIP experiments because of technical problems (see Discussion) I decided to test whether the p53RE2 played an important role in the repression. In order to do this, the site was mutated, as shown in Figure 4.9, using site directed mutagenesis.

p53 Responsive Element 2 mutation

Wild Type	3001	ggccaacatg gtgaaacctc ttctctacta aaattacaaa aattagccgg gcatggtggg
	3061	gcatgcttgt aattccagct
Mutated	3001	ggccaacatg gtgaaacctc ttctctacta aaattacaaa aattagccgg gcatggtggg
	3061	ggatgcttgt aattccagct

Figure 4.9: p53 Responsive element 2 (p53RE2) mutation of the *PLK1* promoter/pGL3 plasmid

The *PLK1* promoter was mutated at three residues (blue) in the p53 RE2 (red).

The mutated plasmid was then transfected into HCT116 p53-null cells, in the same manner as the previous Luciferase plasmid transfections, and Luciferase assays were performed. As shown in Figure 4.10 (A), as p53 levels increased the levels of Luciferase expression from the wild type promoter decreased in a manner similar to that seen in Figure 4.7 (A). However, when the mutant *PLK1* promoter was used, the basal level of expression was much lower suggesting that we had inadvertently changed an activator binding site that is important for full activity. The data also suggested that there was still some level of p53 responsiveness. One trivial explanation for the loss of basal activity is that undesired mutations were introduced elsewhere during the mutagenesis reaction. To check this, the plasmid was sequenced from the beginning of the promoter to the end of the Luciferase gene. Sequencing results showed that no other mutation was introduced into the plasmid (data not shown). Additionally I remade the mutant plasmid from the original plasmid a second time. This second independently-mutated plasmid gave identical results to the first one.

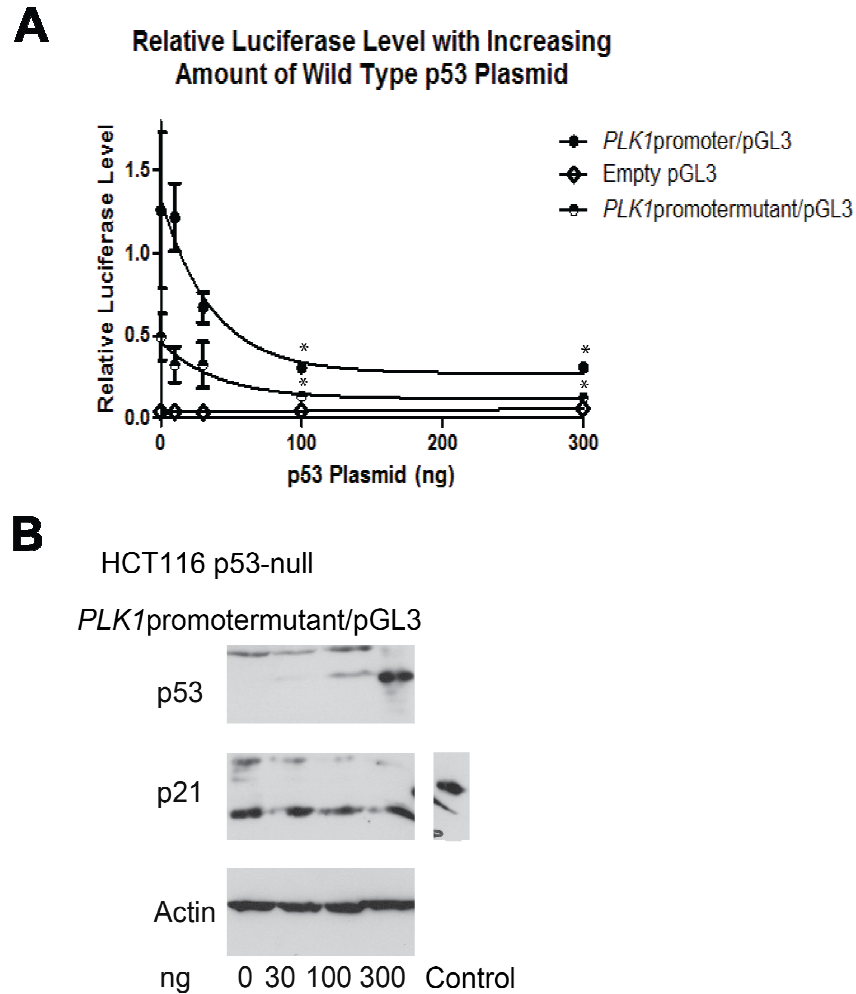


Figure 4.10: Relative Luciferase levels of transfected *PLK1*promotermutant/pGL3 in the presence of increasing amounts of p53 in HCT116 p53-null cells

A) The p53 plasmid was transfected together with *Renilla* plasmid and either the *PLK1*promoter/pGL3 or *PLK1*promotermutant/pGL3 plasmid or empty pGL3 plasmid and also empty PCDNA3 plasmid into HCT116 p53-null cells. After 24 h cells were lysed and a Luciferase assay carried out on the lysates. Luciferase activity levels were then read by a luminometer. Variations in transfection efficiencies were corrected by determining the ratio of activity of Luciferase to *Renilla*. This graph is representative of 3 independent experiments. Statistical analysis was performed using analysis of variance with post hoc Bonferroni test for multiple comparisons (* $p < 0.05$). B) HCT116 p53-null cells were transfected with wild type p53 plasmid together with *PLK1*promotermutant/pGL3, *Renilla* plasmid and empty PCDNA3 plasmid. Lysates were then analysed by Western blotting with anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies.

Figure 4.10 (B) shows that p53 protein levels increase dose-dependently. It was not possible to detect p21 levels under these conditions.

One caveat of using the HCT116 cells is that they may arrest at G1/S in response to p53 expression. For this reason, therefore, the Luciferase transfections were carried out in SAOS2 cells. SAOS2 cells are p53 and Rb null and as a result cannot be inhibited at the G1/S checkpoint. Thus if repression still occurs in these cells, arrest at G1/S can be ruled out as the mechanism of repression. As shown in Figure 4.11 (A), increasing p53 levels caused a corresponding decrease in levels of expression of Luciferase via the wild type *PLK1* promoter. Likewise, similar to experiments in the HCT116 p53-null cells, mutant p53 plasmids were also transfected into SAOS2 cells. As shown in Figure 4.11 (B), the R175H mutant does not cause repression of Luciferase via the *PLK1* promoter. Similarly, the L22Q/W23S mutant (Figure 4.11 (C) and (D)) also do not cause repression of Luciferase. However, notably, the R273H mutant causes a significant increase in the levels of Luciferase. This mutant has been found by other authors to bind to target promoters in a gain of function capacity (Di Agostino *et al.*, 2006).

In conclusion, repression of the expression of Luciferase via the *PLK1* promoter is dependent on wild type p53 and is unlikely to be caused simply by halting the cells at a point in the cell cycle where PLK1 protein levels are low.

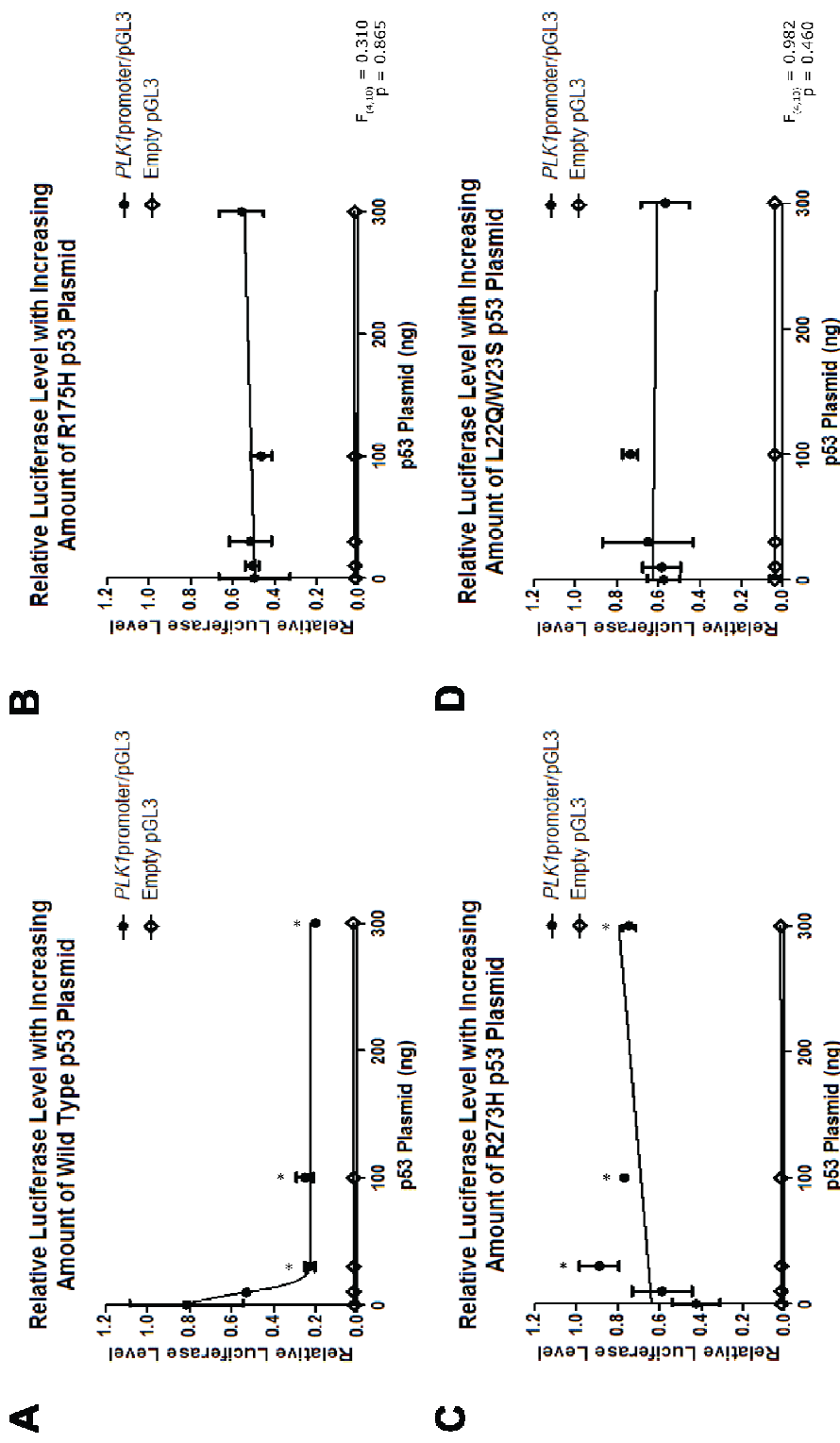


Figure 4.11: Relative Luciferase levels in the presence of increasing amounts of p53 and p53 mutants R175H, R273H and L22Q/W23S in SAOS2 cells

p53 plasmids were transfected together with *Renilla* plasmid, *PLK1*/promoter/pGL3 plasmid or empty pGL3 plasmid and empty PCDNA3 plasmid into SAOS2 cells. After 24 h cells were lysed and a Luciferase assay carried out on the lysates. Luciferase activity levels were then read by a luminometer. Variations in transfection efficiencies were corrected by determining the ratio of activity of Luciferase to *Renilla*. These graphs are representative of 4 independent experiments. Statistical analysis was performed using analysis of variance with post hoc Bonferroni test for multiple comparisons (* $p < 0.05$).

To demonstrate that the p53 plasmid had been transfected into SAOS2 cells and levels of p53 increased with increasing amounts of p53 plasmid, Western blots were performed (Figure 4.12). This Figure confirms that p53 levels increase dose-dependently.

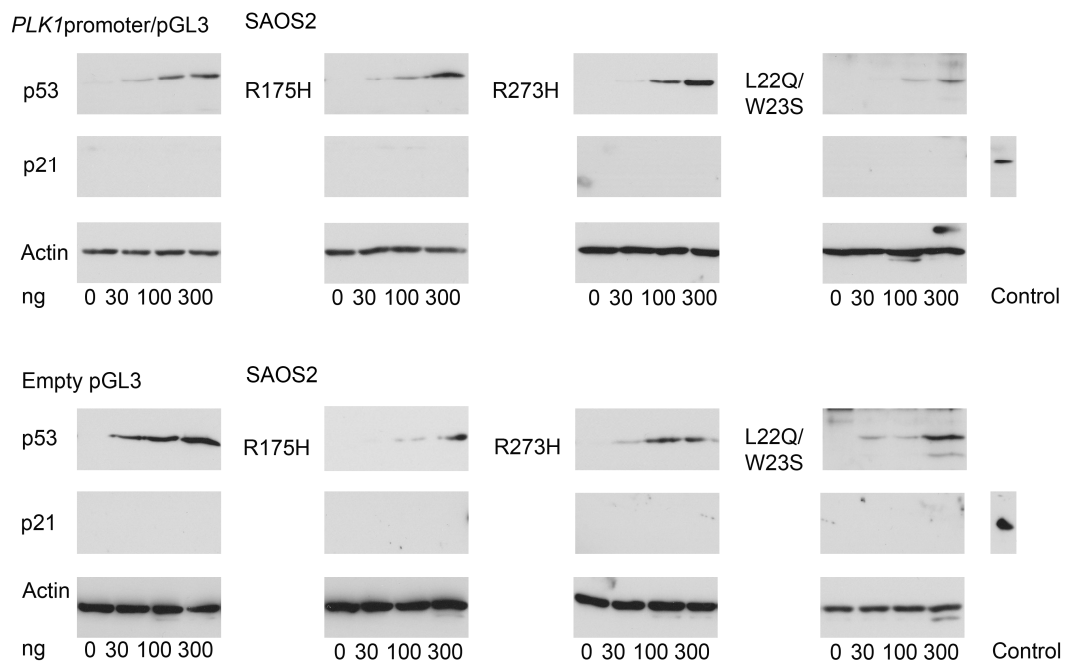


Figure 4.12: p53 levels increase in a dose-dependent manner

SAOS2 cells were transfected with either wild type p53 plasmid or mutant p53 plasmids R175H, R273H or L22Q/W23S together with *PLK1*promoter/pGL3 plasmid or empty pGL3, *Renilla* plasmid and empty PCDNA3 plasmid. Lysates were then analysed by Western blotting with anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies.

Again, similar to experiments carried out in the HCT116 p53-null cells, SAOS2 cells were also transfected with increasing amounts of p53 plasmid together with the *PLK1*promotermutant/pGL3 vector. Similar to experiments in HCT116 cells (Figure 4.10) the basal level of expression was much lower (Figure 4.13).

Figure 4.13 (B) confirms that the p53 plasmid was transfected into the SAOS2 cells and p53 protein levels increased dose dependently.

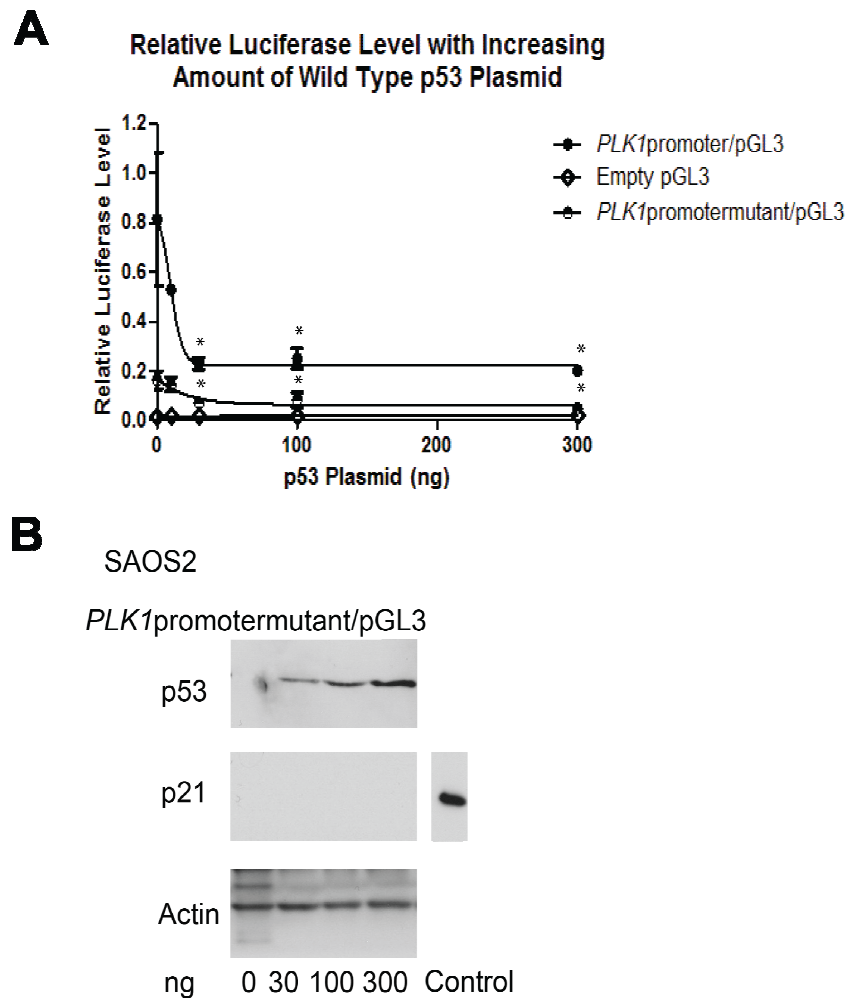


Figure 4.13: Relative Luciferase levels of transfected *PLK1*promotermutant/pGL3 in the presence of increasing amounts of p53 in SAOS2 cells

A) The p53 plasmid was transfected together with *Renilla* plasmid and either the *PLK1*promoter/pGL3 or *PLK1*promotermutant/pGL3 plasmid or empty pGL3 plasmid and also empty PCDNA3 plasmid into SAOS2 cells. After 24 h cells were lysed and a Luciferase assay carried out on the lysates. Luciferase activity levels were then read by a luminometer. Variations in transfection efficiencies were corrected by determining the ratio of activity of Luciferase to *Renilla*. This graph is representative of 4 independent experiments. Statistical analysis was performed using analysis of variance with post hoc Bonferroni test for multiple comparisons (* $p < 0.05$). B) SAOS2 cells were transfected with wild type p53 plasmid together with *PLK1*promotermutant/pGL3, *Renilla* plasmid and empty PCDNA3 plasmid. Lysates were then analysed by Western blotting with anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies.

As discussed in the previous Chapter, p21 also has a role to play in the down-regulation of various cell-cycle regulated proteins. It is therefore possible that the

repression of PLK1 is actually mediated by p21 and that p53 plays an indirect role by stimulating p21 levels. To test this idea, *PLK1*/promoter/pGL3 plasmid was transfected into SAOS2 cells, together with increasing amounts of a p21 expressing plasmid in the absence of p53.

As shown in Figure 4.14 (A), low levels of p21 plasmid transfected into SAOS2 cells do not repress the expression of Luciferase via the *PLK1* promoter. However, at significantly higher levels of p21 plasmid (300 ng) the data suggest that p21 can indeed repress the expression of Luciferase.

Figure 4.14 (B) confirms that p21 was transfected into SAOS2 cells and p21 protein levels increased dose dependently.

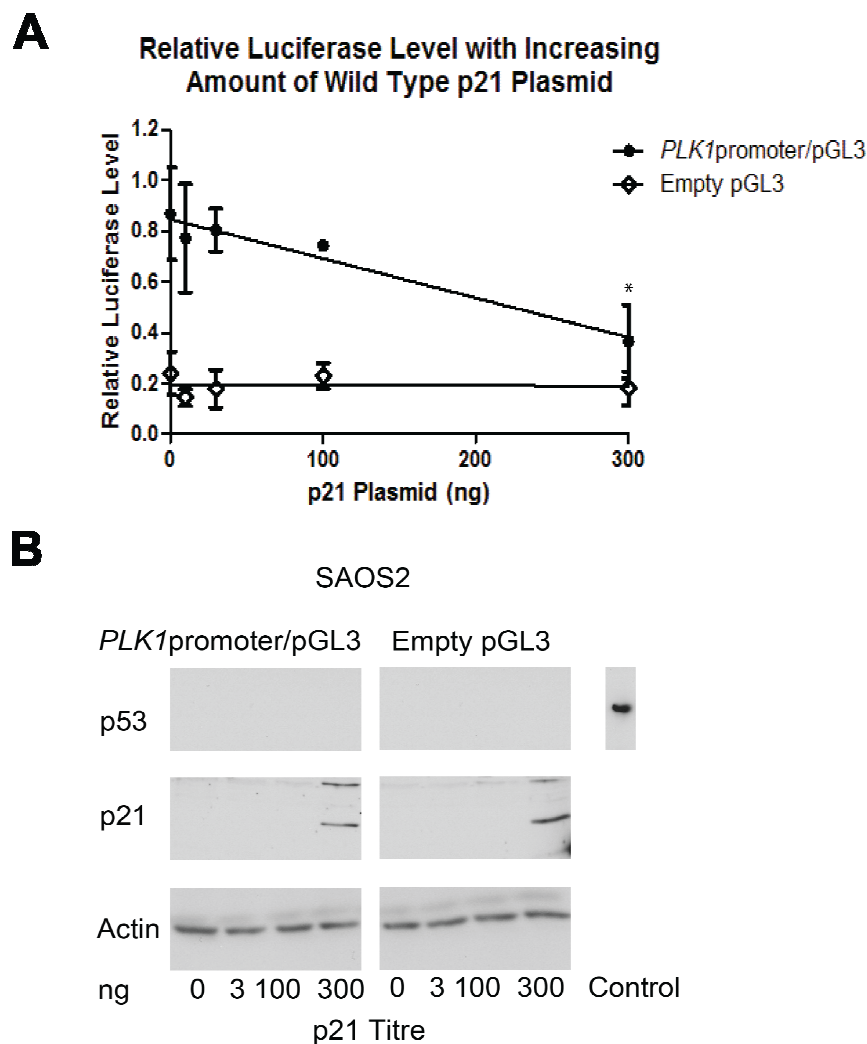


Figure 4.14: Relative Luciferase levels of transfected *PLK1*promoter/pGL3 in the presence of increasing amounts of p21 in SOAS2 cells

A) The p21 plasmid was transfected together with *Renilla* plasmid and either the *PLK1*promoter/pGL3 or empty pGL3 plasmid and also empty PCDNA3 plasmid into SAOS2 cells. After 24 h cells were lysed and a Luciferase assay carried out on the lysates. Luciferase activity levels were then read by a luminometer. Variations in transfection efficiencies were corrected by determining the ratio of activity of Luciferase to *Renilla*. This graph is representative of 3 independent experiments. Statistical analysis was performed using analysis of variance with post hoc Bonferroni test for multiple comparisons (* $p < 0.05$). B) SOAS2 cells were transfected with p21 plasmid together with *PLK1*promoter/pGL3 or pGL3 plasmid, *Renilla* plasmid and empty PCDNA3 plasmid. Lysates were then analysed by Western blotting with anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies.

These results show that p21 may have a part to play in the repression of PLK1.

Although unclear at this stage, p21 repression may be mediated through many pathways, such as the repression through the E2F1 binding site (Shiyanov *et al.*,

1996) or the CDE/CHR element (Uchiumi *et al.*, 1997). p21 represses many genes through the CDE/CHR element in the gene promoter (Kidokoro *et al.*, 2008, Taylor *et al.*, 2001a, Zhu *et al.*, 2002).

To determine whether p53 causes PLK1 repression through the CDE/CHR element, a mutagenesis reaction was carried out in which both the CDE and CHR elements in the *PLK1* promoter of the *PLK1*promoter/pGL3 plasmid were mutated, as shown in Figure 4.15.

CDE/CHR element mutations

Wild Type	3841 cgcgcaggct tttgtaacgt tcccagcgc gcgtttgaat
Mutated	3841 cgcgcaggct tttgtaacgt tcccatatcc gcgttcacat

Figure 4.15: CDE/CHR element mutations of the *PLK1*promoter/pGL3 plasmid

The *PLK1* promoter was mutated at certain residues (blue) in the CDE/CHR element (purple).

This plasmid was then transfected into SAOS2 cells and Luciferase assays carried out. As shown in Figure 4.16 (A), the double mutant *PLK1* promoter plasmid expresses higher levels of Luciferase compared to the wild type *PLK1* promoter plasmid, consistent with published data indicating that this element mediates cell cycle dependent repression of PLK1 (Uchiumi *et al.*, 1997). In the presence of increasing amounts of transfected p53 plasmid, the levels of Luciferase expressed from the *PLK1*promoterCDE/CHRmutant/pGL3 plasmid decreases. In conclusion repression by p53 does not occur through the CDE/CHR element and it is likely p53 induced p21 does not repress PLK1 expression through the CDE/CHR element.

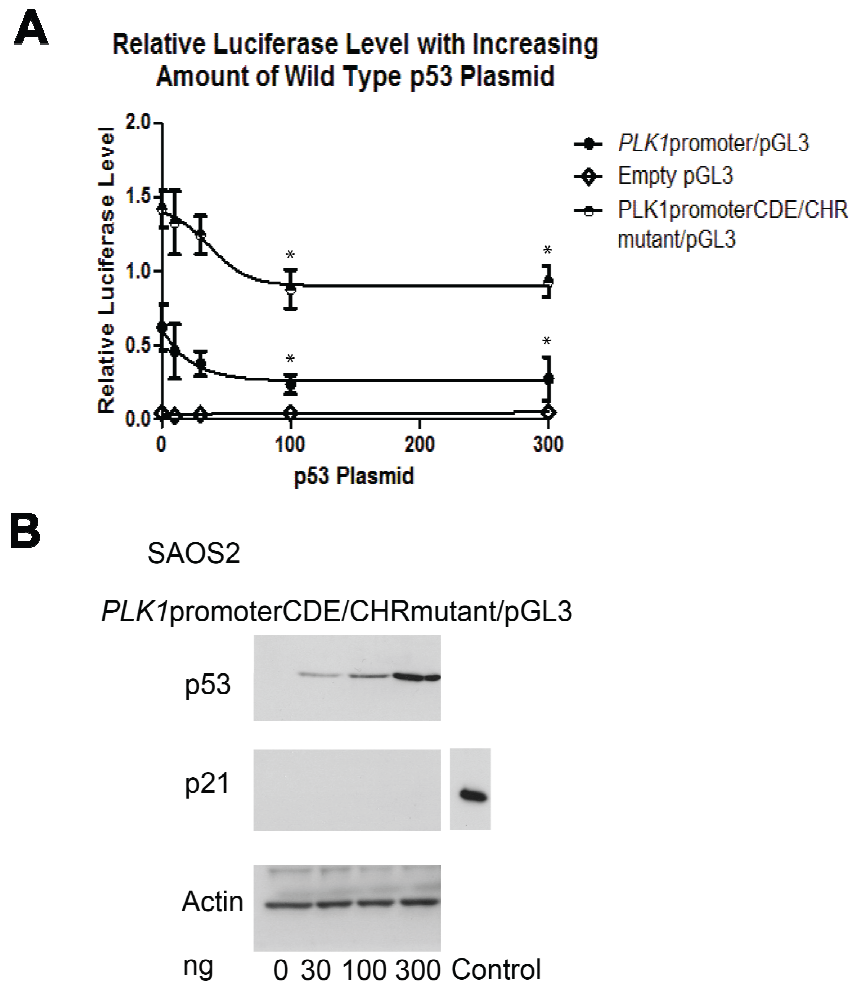


Figure 4.16: Relative Luciferase levels of transfected *PLK1*promoterCDE/CHRmutant/pGL3 plasmid in the presence of increasing amounts of p53 in SAOS2 cells

A) The p53 plasmid was transfected together with *Renilla* plasmid, *PLK1*promoter/pGL3 plasmid, *PLK1*promoterCDE/CHRmutant/pGL3 or empty pGL3 plasmid, and empty PCDNA3 plasmid into HCT116 p53 null cells. After 24 h cells were lysed and a Luciferase assay carried out on the lysates. Luciferase activity levels were then read by a luminometer. Variations in transfection efficiencies were corrected by determining the ratio of activity of Luciferase to *Renilla*. This graph is representative of 3 independent experiments. Statistical analysis was performed using analysis of variance with post hoc Bonferroni test for multiple comparisons (* $p < 0.05$). B) SAOS2 cells were transfected with p53 plasmid together with *PLK1*promoterCDE/CHRmutant/pGL3 plasmid, *Renilla* plasmid and empty PCDNA3 plasmid. Lysates were then analysed by Western blotting with anti-p53 antibodies, anti-p21 antibodies and anti- β -Actin antibodies.

These data overall suggest that neither p53 nor p21 repress the expression of PLK1 through the CDE/CHR element in the *PLK1* promoter, however, it does not exclude the repression of PLK1 through any other domains. Therefore future mutagenesis

experiments of these domains may shed light on the mechanism of PLK1 repression by p53 and p21. ChIP data from our collaborators suggest that p53 does not interact with any of the other elements in the core promoter region.

4.5 p53 binds at the p53 responsive element 2

To determine, *in vitro*, whether p53 binds to the proposed p53 responsive element 2, an electrophoretic mobility shift assay (EMSA) was carried out. As described in Chapter 2, oligo-nucleotides containing the wild type or mutated sequence of the p53 responsive element were incubated in the presence or absence of p53. Using this method we are able to detect the p53 bound oligo-nucleotide (probe). To further show that it is indeed p53 that has bound to the oligo-nucleotide, the antibody PAb421 was added (Hupp *et al.*, 1992). This particular antibody activates the cryptic site specific DNA binding activity of p53 (Hupp *et al.*, 1992), therefore enhancing the site specific DNA binding, as measured by a higher intensity of the p53 bound peptide in the gel (Figure 4.17). As shown in the Figure, p53 binds to the wild type oligonucleotide probe, however p53 binding to the mutated oligo-nucleotide is reduced. In the presence of PAb421 the complex has been super-shifted and the site-specific DNA binding of p53 to the oligo-nucleotide has increased as shown by the intensity of the band. However, there is a decrease of p53 binding to the mutated oligo-nucleotide in the presence of the antibody compared to the wild type oligo-nucleotide. p53 binds to the control TL oligo-nucleotide, but in the presence of the antibody there is no increase in intensity of the band indicating that there is no increase in DNA binding and DNA binding of p53 to the TL oligo-nucleotide is unspecific, as expected. On the other hand, p53 binds only to the PG oligo-

nucleotide in the presence of the antibody indicating that there is only site-specific binding of p53 to the PG oligo-nucleotide, as expected.

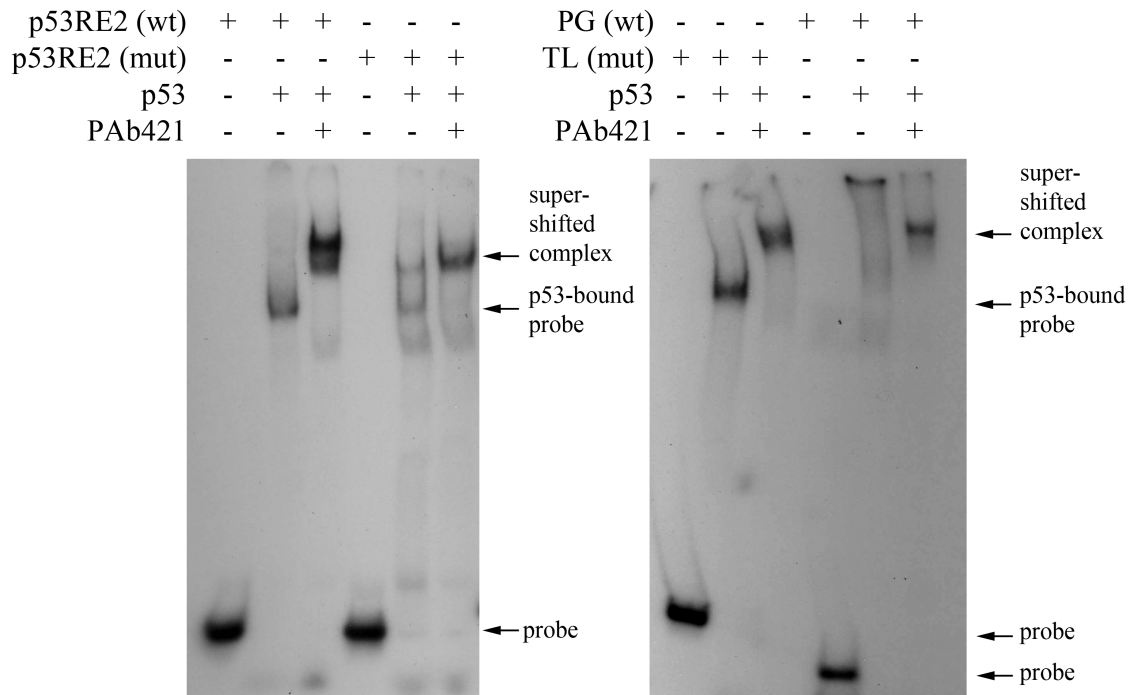


Figure 4.17: Electro-phoretic mobility shift assay shows p53 binds to the PLK1 promoter DNA and binds with a lower specificity to the mutant PLK1 promoter DNA

Electro-phoretic –mobility shift assay. Labelled oligo-nucleotides were incubated in the absence and presence of p53 protein, which were themselves incubated in the absence or presence of the antibody, PAb421 and run on a non-SDS PAGE gel. (n= 4).

These results show that p53 binds to the wild type oligo-nucleotide with more affinity than the mutated oligo-nucleotide and further activation of the site-specific DNA binding activity of p53 confirms this. Overall this suggests that p53 is indeed binding to the p53 responsive element 2, *in vitro*.

4.6 p53 levels increase on the p53 responsive element after DNA damage

To elucidate whether p53 binds to the p53 responsive element *in vivo*, semi-quantitative chromatin immunoprecipitations (ChIP) were carried out by our collaborators (Katie Schumm, Sam Nicol). Their results showed that there was no p53 at the CAATT box or at the p53 RE1. Additionally, they showed that p53 is present at the p53 RE2 and increases when cells are treated with DNA damaging agents. Interestingly there was a decrease in the levels of acetylated histone-3 when cells are treated with DNA damaging agents. Furthermore, controls indicated that there was no p53 present at the GAPDH promoter. There were no changes in the levels of acetylated histone-3 at the GAPDH promoter.

However, to determine whether Nutlin-3 causes an increase in the levels of p53 at this region, further ChIP experiments and PCR reactions were carried out by myself. As shown in Figure 4.18, the levels of p53 at the p21 promoter increase in the presence of Etoposide or Nutlin-3, as expected, and no background DNA was picked up in the no antibody PCR reaction. To determine whether there are increases in p53 binding to the *PLK1* promoter, further ChIP experiments and PCR reactions were carried out and show that there was a high background of DNA in the no antibody controls. Unfortunately, despite making many individual changes to the ChIP protocol, we were unable to reduce the background DNA to a level that permitted unequivocal interpretation of data. The conditions used were sufficient to give reproducible data when analysing the *p21* gene. One additional issue with the PLK1 promoter was finding the most suitable primer pair to provide confidence in the ChIP data.

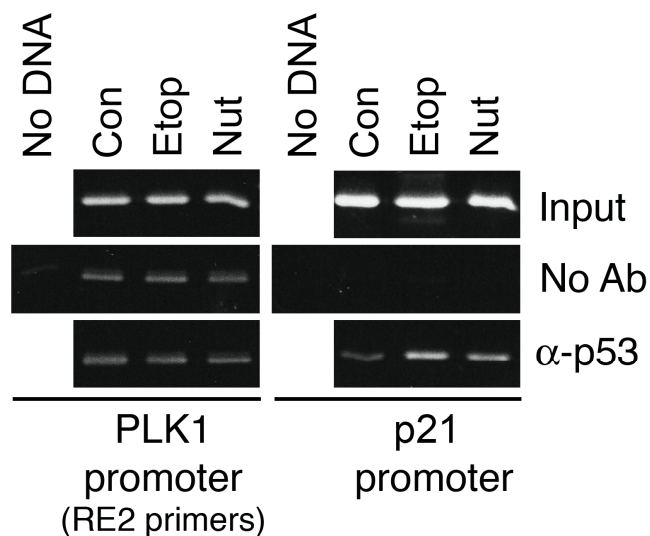


Figure 4.18 Chromatin Immunoprecipitations show that levels of p53 at the p21 promoter increase after DNA damage

ChIP was carried out and PCR was performed to show whether p53 was present at p53 RE2 on the PLK1 promoter and whether p53 was present on the p21 promoter.

Our results suggest that the ChIP protocol was efficient, however I was unable to conclude whether p53 binds to the p53RE2, using this method. However, since the writing of this thesis and the technical advances in the ChIP technique in our lab during this time, members of our lab show quantitatively that Nutlin-3 increases the amount of p53 at RE2.

4.7 Concluding Remarks

When addressing whether the *PLK1* promoter was repressed by p53 through proposed p53 responsive elements, it was necessary to clone the PLK1 promoter into a reporter vector. Using HCT116 p53-null cells and SAOS2 we created a system where the PLK1 promoter reporter vector could be transfected together with increasing amounts of either p53 or p21 and determine whether repression was

caused by either protein. Our data show that p53 is able to repress PLK1 and p21 may have a small part to play in this repression.

Furthermore, our system allowed us to determine where within the PLK1 promoter repression was achieved and rules out other domains which might occur through, such as the CDE/CHR element (Lucibello *et al.*, 1995, Uchiumi *et al.*, 1997). Future study will reveal whether repression occurs through other as yet undefined domains. Our data show that p53 carries out repression of PLK1 at the proposed p53 responsive element 2.

To further elucidate the mechanism by which p53 represses *PLK1* promoter at this element, EMSA was carried out and confirmed that p53 binds to p53 RE2. This suggested that the mechanism by which p53 mediates repression is through its transcription factor ability, confirmed by mutant p53 in the Luciferase experiments.

ChIP, carried out by our collaborators, further confirm that p53 directly represses PLK1 through the p53 RE2. Overall the results in this Chapter show that there is a repression mechanism of PLK1 that is dependent on p53 and partly on p21.

Chapter 5: Conclusions and Discussion

p53 is a well characterised transcription factor that is activated in response to a range of cellular stresses. A variety of genes that are transcriptionally activated by p53 have been studied in depth including *p21*, *14-3-3* and *GADD45* (Riley *et al.*, 2008). However, gene profiling studies have indicated that most targets of p53 are actually repressed by p53 (Kho *et al.*, 2004, Riley *et al.*, 2008). This thesis shows that PLK1, which is essential for progression of the cell cycle in G2 and M, is an important and significant target for p53 mediated repression and p53 regulated G2/M arrest.

Previous to this thesis, we (Dr Sylvia Dias, data not published) and others (Ando *et al.*, 2004, Kho *et al.*, 2004) showed that PLK1 protein levels are down-regulated in response to DNA damage. My studies, described in this thesis, explore the mechanisms of PLK1 down-regulation by DNA damage and show that p53 is both necessary and sufficient to mediate this effect not only in response to DNA damage but also to other stimuli that induce p53. These observations were made following treatments in the well characterised human colon cancer cell line, HCT116 and its p53-null derivative (Bunz *et al.*, 1998) with three different types of drug: Etoposide, which causes DNA double strand breaks, Cisplatin, which promotes the formation of DNA adducts and Nutlin-3, which activates p53 in a non-DNA damage dependent manner by inhibiting the interaction with its negative regulator, MDM2. The observation that Nutlin-3 can stimulate down-regulation of PLK1 in the absence of a DNA damage stimulus suggests that well-characterised stimuli that induce p53 independently of DNA damage (e.g. oncogenes, hypoxia) are also likely to down-regulate PLK1 levels and show a similar biological outcome. This however remains to be tested experimentally.

My results also show that mRNA levels are down-regulated following induction of p53 in a non-DNA damage dependent manner. These data are consistent with the idea that p53 can regulate PLK1 mRNA levels either by a transcriptional mechanism or perhaps through changes in mRNA turnover. The data from the Luciferase experiments (discussed below) strongly favour a mechanism that involves p53-mediated repression of the PLK1 promoter.

This thesis has not only dissected the direct repression of PLK1 by p53 but has also taken into consideration the possibility that p53 can indirectly down-regulate PLK1. PLK1 expression is under tight control throughout the cell cycle due to the presence of a CDE/CHR repression element located within the PLK1 promoter (Uchiumi *et al.*, 1997) . Thus PLK1 is found at low levels during G1 and S phase but levels increase in G2 and peak in mitosis, due to cell cycle mediated relief of this repression. During stressed conditions p53 induces cell cycle arrest at the G1/S checkpoint by activating expression of the CDK1 inhibitor, p21 (el-Deiry *et al.*, 1993, Weinberg and Denning, 2002) and in doing so the cell cycle arrests at a point where PLK1 levels are at their lowest (G1/S). To confirm that PLK1 levels cycle in the HCT116 cell line, as has been observed in other cell lines, a double Thymidine block and release was carried out (Figure 3.3). This experiment verified that PLK1 levels are cell cycle regulated in HCT116 cells and raised the issue of whether the drug treatments were simply causing a cell cycle arrest at a stage where PLK1 levels are low. Therefore to eliminate possible indirect p53 cell cycle regulated repression of PLK1, two experiments were carried out. In the first, p21 expression (p21 mediates the G1/S arrest) was silenced in HCT116 cells and in U2OS cells. Subsequent induction of p53 still led to down-regulation of PLK1 levels suggesting that p21 (and therefore G1/S arrest) was not sufficient to mediate this effect.

However the down-regulation of PLK1 was slightly less efficient suggesting that p21 may contribute to this effect. In the second approach, HCT116 p21-null derivatives were treated with each of the drugs used to induce p53. This analysis confirmed that Nutlin-3 was still able to down-regulate PLK1 but only weakly when compared with the parental cells. Down-regulation in response to Etoposide or Cisplatin was lost (but interestingly not at earlier time points). Analysis of the treated and untreated HCT116 cells by flow cytometry confirmed that neither the p53-null nor the p21-null derivatives were able to undergo a G1/S arrest yet PLK1 levels could still be down-regulated. While these observations indicate repression of PLK1 by p53 can still occur in the absence of p21, the induction of p53 and p53 down-stream gene expression in the HCT116 p21-null derivatives was very weak. It is therefore impossible to gain any further useful information about any specific role that p21 may play in PLK1 regulation from the use of this cell line.

To examine the involvement of p53 directly on PLK1 repression, Luciferase reporter assays were carried out and results show that wild type p53 directly represses PLK1 through the promoter. This experiment involved the construction of a plasmid which allowed expression from the PLK1 promoter to be assessed *in vivo*, in the presence of increasing quantities of a p53 expressing plasmid. The titration of mutant forms of p53, which either have lost the ability to bind to DNA or have alterations in the N-terminal domain (which is normally required for trans-activation but it is also thought to play a role in trans-repression (Hammond *et al.*, 2006)) did not lead to PLK1 repression. These results confirm that wild type p53 alone is sufficient to repress PLK1 and that repression requires both the N-terminal domain and site-specific DNA binding activities of p53.

In order to determine any possible contribution of p21 in a transcriptional capacity, p21 expressing plasmid was titrated into cells for the Luciferase experiment. The results show that p21 can repress PLK1 expression but to a smaller extent to that of p53. Technically, it was extremely difficult to show whether the levels of expression of p21 achieved using the p21 plasmid matched those obtained from endogenous p21 expression following transfection of the p53 plasmid. The extent of any contribution of p21 towards inhibiting PLK1 expression therefore remains uncertain.

Independently of its ability to induce G1/S block, this thesis clearly shows p53 represses PLK1 directly, therefore the next aim was to discover where on the PLK1 promoter p53 was able to carry out its repression function. Earlier reports described that some promoters such as the Survivin and CDC25C promoters were regulated by p53 through p53 responsive elements (Hoffman *et al.*, 2002, St Clair *et al.*, 2004) and this led us to look for similar elements in the PLK1 promoter. We identified (with the help of our colleague Dr Jean Christophe Bourdon) two possible p53 responsive elements in the PLK1 promoter, termed RE1 and RE2. To find out whether p53 binds to the p53 RE2 (the more compact of these two sites) *in vitro*, EMSA was carried out. This analysis confirmed that there is sequence specific binding of p53 to this site, as mutation of this site decreased binding of p53. To determine whether p53 could repress PLK1 through the p53 responsive element(s), p53 RE2 on the PLK1 promoter of the reporter plasmid was subsequently mutated at a half site in the p53 consensus sequence and was transfected into cells together with increasing amounts of p53 expressing plasmid. Luciferase assays carried out on these cells gave an unexpected result and showed that PLK1 expression was significantly reduced because of this mutation. It was first thought that there may have been a mutation introduced elsewhere into the vector after mutagenesis and

therefore the entire plasmid was sequenced. Sequencing results showed no other mutations in the vector. However, in the event that another mutation was not picked up in the sequencing, the mutagenesis reaction was carried out a second time and the vector transfected into cells. The Luciferase assays showed the same result. After eliminating the possibility that another mutation was introduced that affected Luciferase expression these results suggest that mutation of this site in RE2 causes a disruption to the binding site of an as yet unidentified activator of PLK1 expression and that perhaps part of the mechanism by which p53 represses PLK1 may be through competitive displacement, which is a common mechanism for p53-dependent repression as described by St Clair *et al.*, 2006.

Previous publications also show that p53 and p21 may act to repress gene promoters, such as the CDC25C promoter, through both the p53 responsive element and the CDE/CHR element (St Clair *et al.*, 2004) and that p53 binds directly to this sequence to repress gene expression. As the PLK1 promoter has an already identified CDE/CHR element, described by Uchiumi *et al.*, 1997 it was therefore logical to mutate the CDE/CHR element to elucidate whether p53 may be acting through this site to repress PLK1 expression. Results show that mutation of the CDE/CHR gives rise to a substantial increase in the expression of Luciferase. This was expected as mutation of this domain abrogates repression of PLK1 in both G1 and S phases of the cell cycle. However, contrary to results shown by St Clair *et al.*, 2004, who mutated the CDE/CHR element in the CDC25C promoter and found that mutation reduces repression of CDC25C expression by both p53 and p21, the results here show that mutation of the CDE/CHR element in the PLK1 promoter has no effect on p53 repression of PLK1 expression suggesting that repression of PLK1 expression by p53 is not dependent on the CDE/CHR element.

Given that the results described above suggest that p53 represses PLK1 expression via the RE2 on the PLK1 promoter, I therefore sought to carry out Chromatin immunoprecipitation experiments to find out whether p53 binds directly to this region in the PLK1 promoter and also to rule out the binding of p53 to other sites, such as RE1 and the CDE/CHR element. I also sought to show if levels of p53 at this region increased following the induction of p53 expression by Etoposide and Nutlin-3. As already discussed in Chapter 4, ChIP experiments were carried out by our collaborators (Katie Schumm, Sam Nicol). Their results showed that there was no p53 at the CAATT box or at the p53 RE1. Additionally, they showed that levels of p53 are present at the p53 RE2 and increase when cells are treated with DNA damaging agents. Interestingly there was a decrease in the levels of acetylated histone-3 when cells are treated with DNA damaging agents. Unfortunately, despite making many individual changes to the ChIP protocol, I was unable to remove background DNA which was picked up by the *PLK1* promoter p53 RE2 primers and therefore could not conclude whether there was an increase in p53 levels at the RE2 in the presence of Nutlin-3.

However, new data from our lab (carried out by Sharon King and Lynnette Marcar and obtained due to a significant improvement of the ChIP technology used in the lab) show quantitatively and reproducibly that p53 levels do indeed increase in the presence of Nutlin-3 at the RE2 and that p53 binds to RE1 even in the absence of stimuli such as Nutlin-3 or the DNA damaging agent, Etoposide at low levels. They also confirm that, interestingly, levels of acetyl-histone 3 decrease at RE2 in response to these drugs, indicating a role for PLK1 repression by histone deacetylases.

To confirm the involvement of HDACs in the repression mechanism, HCT116 and their p53-null derivatives were treated with the HDAC inhibitor Trichostatin A (TSA). TSA alone caused PLK1 repression which may be a result of TSA relieving repression of an as yet unknown PLK1 negative regulator. As established earlier, Nutlin-3 almost abolished levels of PLK1 however the presence of both treatments recovered levels of PLK1 to the same level of TSA alone. These results support the idea that p53-mediated down-regulation of PLK1 may occur through a mechanism that involves both p53 and p53 recruited HDAC transcriptional expression.

This thesis demonstrates the direct repression of PLK1 by p53 and rules out cell cycle regulated repression of PLK1 as the major (indirect) mechanism by which p53 can repress expression of this gene. This project identifies two potential and perhaps overlapping mechanisms of repression of PLK1 by p53. These are shown in Figure 5.1.

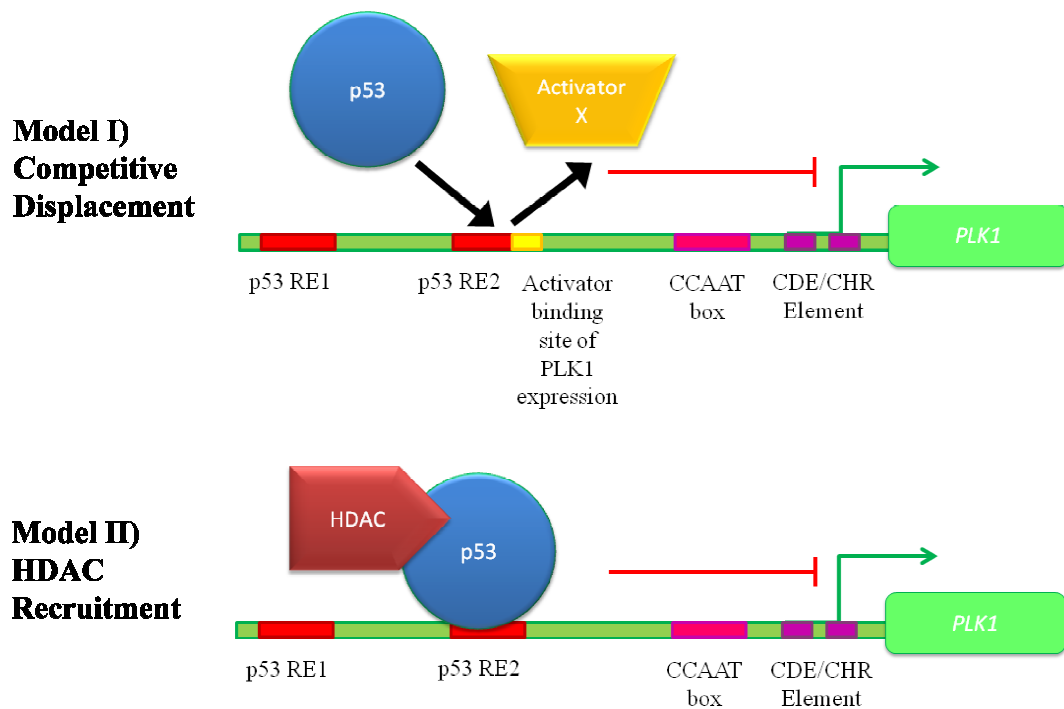


Figure 5.1: Two potential mechanisms of p53 repression of *PLK1*

This thesis suggests two mechanisms by which p53 repress *PLK1* expression. Model I shows p53 could do this through competitive displacement of an activator of *PLK1* expression, Model II shows p53 could do this through the recruitment of HDACs.

This thesis shows that p53 is both necessary and sufficient for down-regulation of PLK1 and advances the understanding by which p53 down-regulates PLK1 that previous publications have suggested or inferred (Ando *et al.*, 2004, Incassati *et al.*, 2006, Kho *et al.*, 2004, Ree *et al.*, 2003, Smits *et al.*, 2000, Sur *et al.*, 2009).

Given the importance of p53 in cell cycle arrest, PLK1 knock-down experiments were subsequently carried out to elucidate whether the simple removal of PLK1 in cells would replicate the results seen in the drug treated cells. Several previous publications show that PLK1 knock-down causes defects in bipolar spindle formation and cytokinesis, growth inhibition, and apoptosis induction in human cancer cell lines (Guan *et al.*, 2005, Liu and Erikson, 2003, Liu *et al.*, 2006, Yim and

Erikson, 2009). These reports also show that p53-null cells are more sensitive to PLK1 depletion and have a higher number of cells undergoing apoptosis. Notably they also show that PLK1 depletion leads to cell death in cancer cells where it does not in normal cells and that the absence of p53 as well as PLK1 leads to cell death of normal cells. The PLK1 siRNA results in this thesis, consistent with these previous studies, show that PLK1 knock-down causes the cells to arrest in mitosis, as shown by flow cytometry, changes in cellular morphology and the presence of high levels of phosphorylated histone 3, a marker of mitosis. There is also an increase in the number of cells in apoptosis shown by the presence of cleaved PARP, a marker of apoptosis. This result shows that PLK1 knock-down does not replicate the results seen with the drug treatments, which show cell cycle checkpoint arrest at G1/S and/or G2/M (the drug treated cells are more likely in G2 rather than M phase as the cell morphology of these cells did not show the rounded up appearance characteristic of mitotic cell when harvested). Interestingly, the number of cells in both mitosis and apoptosis rise dramatically in the absence of p53 or p21.

These results are very intriguing and perhaps the most plausible explanation to why PLK1 knock-down cannot replicate G2/M checkpoint arrest is that p53 actually orchestrates a series of simultaneous events, such as down-regulation of CDC25C and Cyclin B (Stark and Taylor, 2006) as well as PLK1, to cause cell cycle arrest at the G2/M checkpoint and inhibit the cell cycle before it moves into mitosis. The reduction of PLK1 by siRNA causes DNA damage (Yim and Erikson, 2009), severe mitotic catastrophe and inhibition of mitosis and because of this the cells cannot undergo further proliferation and become apoptotic. p53 arrests the cells at the G2/M checkpoint (in addition to the G1/S checkpoint) in the cell cycle before mitotic catastrophe and apoptosis need occur. Furthermore the absence of p53 or p21

accentuates the number of cells in apoptosis and therefore suggests that p53 plays a protective role which is a role also suggested by other authors (Guan *et al.*, 2005, Sur *et al.*, 2009, Yim and Erikson, 2009).

In the overall regulation of the cell cycle this project supports and advances the knowledge of regulation of the cell cycle by p53 and the mechanistic insight by which p53 carries this out, as shown in Figure 5.2.

PLK1 Repression: the model

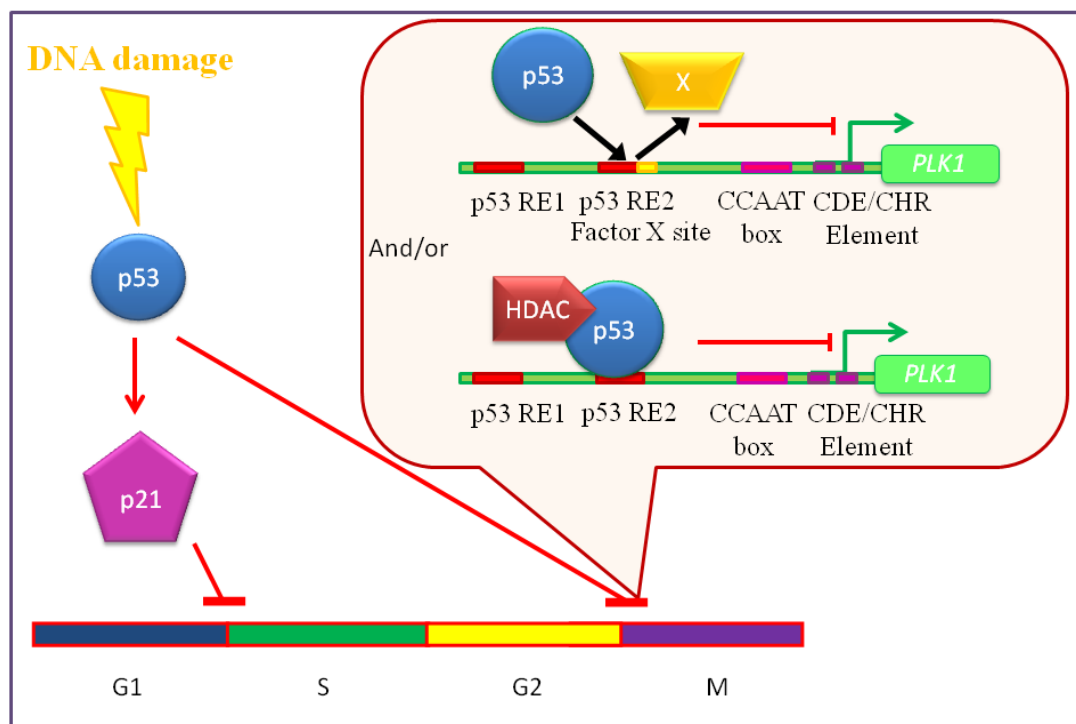


Figure 5.2: Cell cycle regulated and direct repression of PLK1 by p53

Incorporating the two proposed mechanisms by which p53 represses PLK1, this model shows that p53 directly represses PLK1 and mediates the block of the cell cycle at the G2/M checkpoint. However, this diagram does not show the other genes that are trans-activated and repressed as part of the G2/M checkpoint, for example CDC25C and Cyclin B, by p53.

This model shows that there are still questions to answer in regard to the exact mechanism by which p53 represses PLK1. One important question is: are HDACs

recruited to the p53 RE and if so are they recruited to the RE by p53? Imbriano *et al.*, 2005 suggest a model whereby the p53-NFY-CCAAT box complex binds to HDACs to repress gene expression however, they show that this complex is required but not sufficient for HDAC recruitment. ChIP data shown by our collaborators show no occurrence of p53 at the PLK1 promoter CCAAT box, therefore this data suggests that it is unlikely that HDAC recruitment and trans-repression by this mechanism occurs at the PLK1 promoter.

Other authors have suggested that HDACs are recruited to sequence specific DNA-binding transcriptional repressors by the co-repressor protein, mSin3a (Pazin and Kadonaga, 1997). p53 is one such transcriptional repressor that binds to mSin3a which in turn binds to HDACs and together mediate trans-repression (Murphy *et al.*, 1999) (for review see (Ho and Benchimol, 2003)). Some authors even suggest that HDACs may be recruited directly to p53 or through other mediator proteins such as N-CoR or SMRT (Juan *et al.*, 2000, Pazin and Kadonaga, 1997). Taken together these data therefore suggest that a future experiment that could be carried out and that would find out whether HDACs are found at p53RE2, would be to ChIP certain HDACs, to elucidate whether they are recruited to this site. With more time and using methods such as the ChIP re-ChIP method, it may be possible to immunoprecipitate HDACs alongside p53 on the PLK1 promoter and provide insight as to whether this mechanism of repression may occur at the PLK1 promoter.

HDACs are known for the ability to remove acetylation groups from histones but they also mediate the reduction of acetylation of p53, which ultimately inhibits p53 trans-activation (Juan *et al.*, 2000). These authors suggest that there are two mechanisms by which p53 represses gene expression with the use of HDACs: the

first is that HDACs enhance p53 repression of genes by the removal of histone acetylation and the second is that HDACs deacetylate p53, in doing inhibiting trans-activation of gene expression by p53. Recently, Kruse *et al.*, 2009 describe a trans-repression mechanism whereby p53 is held on the DNA in a repressed state due to the binding of MDM2 to p53. Acetylation of p53 activates p53 by inhibiting the interaction of p53 to MDM2 (Li *et al.*, 2002), thereby allowing p53 to activate gene expression. Also contrary to data shown by Juan *et al.*, 2000, other studies show that the binding of mSin3a to p53 stabilises p53 by inhibiting p53 proteasomal degradation by MDM2 (Zilfou *et al.*, 2001). This suggests that HDACs do not inhibit p53 trans-activation nor mediate MDM2 mediated proteasomal degradation of p53.

Taken these data, and the data that show that p53 is found on the PLK1 promoter regardless of stress (EMSA and ChIP), together, could it be that the mechanism of repression by p53 is the simple removal of MDM2 from p53 and that this would allow the mSin3a-HDAC complex to bind to p53 and mediate p53 repression of PLK1? This might explain why Nutlin-3 can induce PLK1 repression and reduce PLK1 levels so effectively compared to the two other p53 activating agents described in this thesis. Given that DNA damage leads to the phosphorylation and destruction of MDM2, this model would also fit with the observations that Etoposide and Cisplatin can also repress PLK1.

In respect with the competitive displacement model, experiments to identify the activator of PLK1 expression found at p53RE2 would be ideal to ascertain whether p53 competes with this protein, causing transcriptional repression, and would support the model that competitive displacement contributes to the mechanism by

which p53 represses PLK1, as suggested by St Clair *et al.*, 2004 at the CDC25C promoter.

As to other sites located in the PLK1 promoter, to support the data that suggest that the CCAAT box is not involved in p53 repression, perhaps mutation of the CCAAT box and Luciferase assays will confirm that p53 repression is independent of the CCAAT box and rule out this possible mechanism of repression of PLK1 by p53. However, mutation of the CCAAT may eliminate expression of PLK1 altogether. It may also be interesting to mutate the E2F binding site to rule out possible p21 involvement in PLK1 repression at this site.

PLK1 knock-down is a potential therapeutic approach to cancer as suggested by data that show that cancer cells are more sensitive to knock-down than normal cells and the induction of mitotic arrest and apoptosis is further increased in cells with defective p53 (Guan *et al.*, 2005, and as data in this thesis show). The presence of p53 in normal cells clearly gives these cells a level of protection when PLK1 is knocked down or inhibited by inhibitors, whilst p53 defective cancer cells treated with PLK1 inhibitors will undergo apoptosis. Thus PLK1 inhibition or knock-down provides a selective treatment to cancer cells.

The data in this thesis support the model that p53 has a substantial effect on the levels of PLK1. Some authors even suggest that in the absence of p53, levels of PLK1 increase (Sur *et al.*, 2009), although my own data comparison of parental and p53-null HCT116 cells do not support this. This project provides a model of the mechanism by which p53 regulates PLK1 and how mutation or loss of p53 in tumour cells can contribute towards unregulated PLK1 levels in these cells and therefore an increase in proliferation of cancer cells.

The work described in this thesis further expands our understanding of the mechanism by which PLK1 is deregulated in cancer cells and the role p53 has in regulating PLK1 in normal cells. Mutation of p53 in cancer cells prevents PLK1 repression and therefore allows cancer cell proliferation. Targeting and inhibiting PLK1 could therefore be utilised in cancer therapy. The clinical potential of PLK1 inhibitors in cancer therapy is an active area of research and PLK1 inhibitors are already in early clinical development. These include BI 2536, BI 6727, GSK461364 and HMN-214 (Schoffski, 2009). Some are also in advanced preclinical development, for example NMS-1, CYC-800, DAP-81, and LC-445.

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Appendix

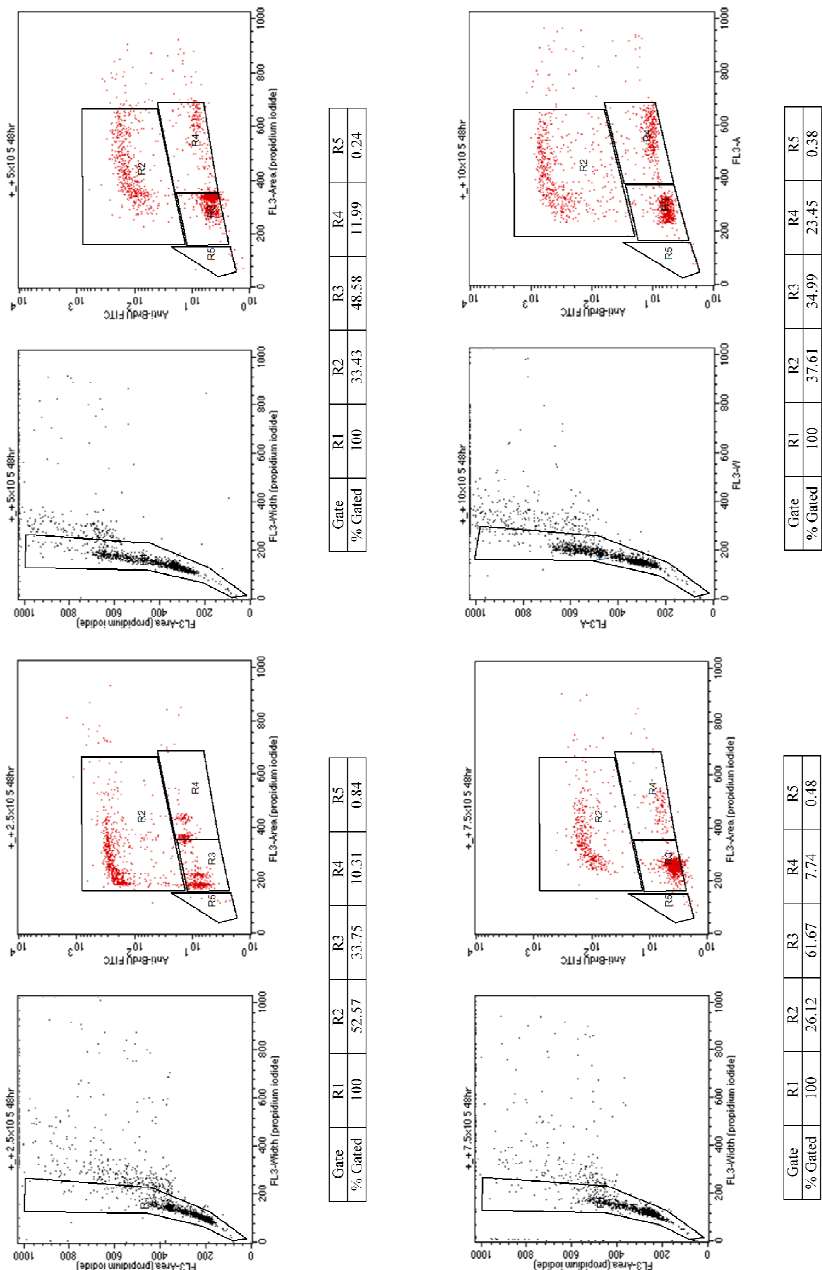


Figure A1: Flow cytometry analysis of HCT116 wild type cells at increasing seeding densities

HCT116 wild type cells were seeded at densities of 2.5x10⁵, 5x10⁵, 7.5x10⁵ and 1x10⁶ and put into the incubator for 48 h. Cells were then harvested, as described in Chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content, and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

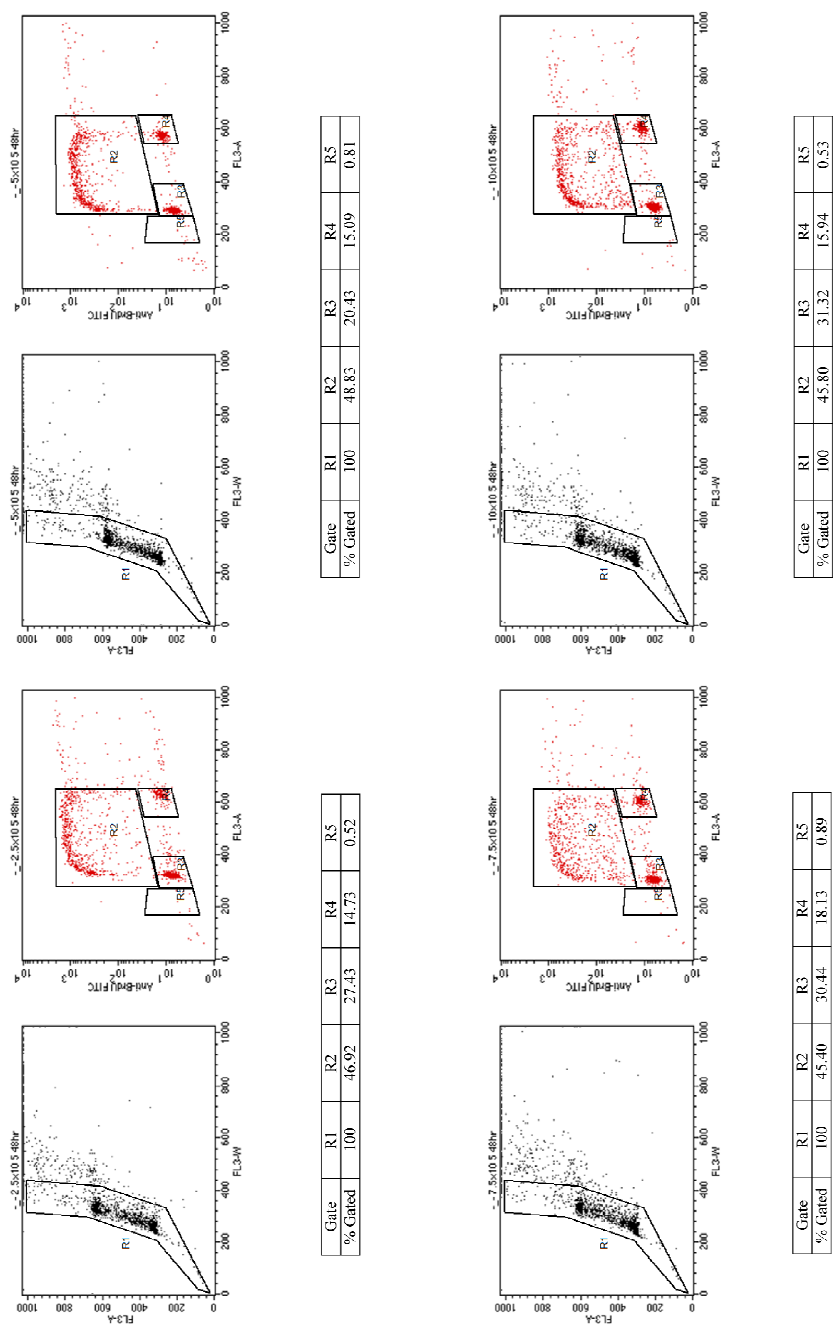


Figure A2: Flow cytometry analysis of HCT116 p53-null cells at increasing seeding densities

HCT116 p53-null cells were seeded at densities of 2.5×10^5 , 5×10^5 , 7.5×10^5 and 10×10^5 and put into the incubator for 48 h. Cells were then harvested, as described in Chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content, and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

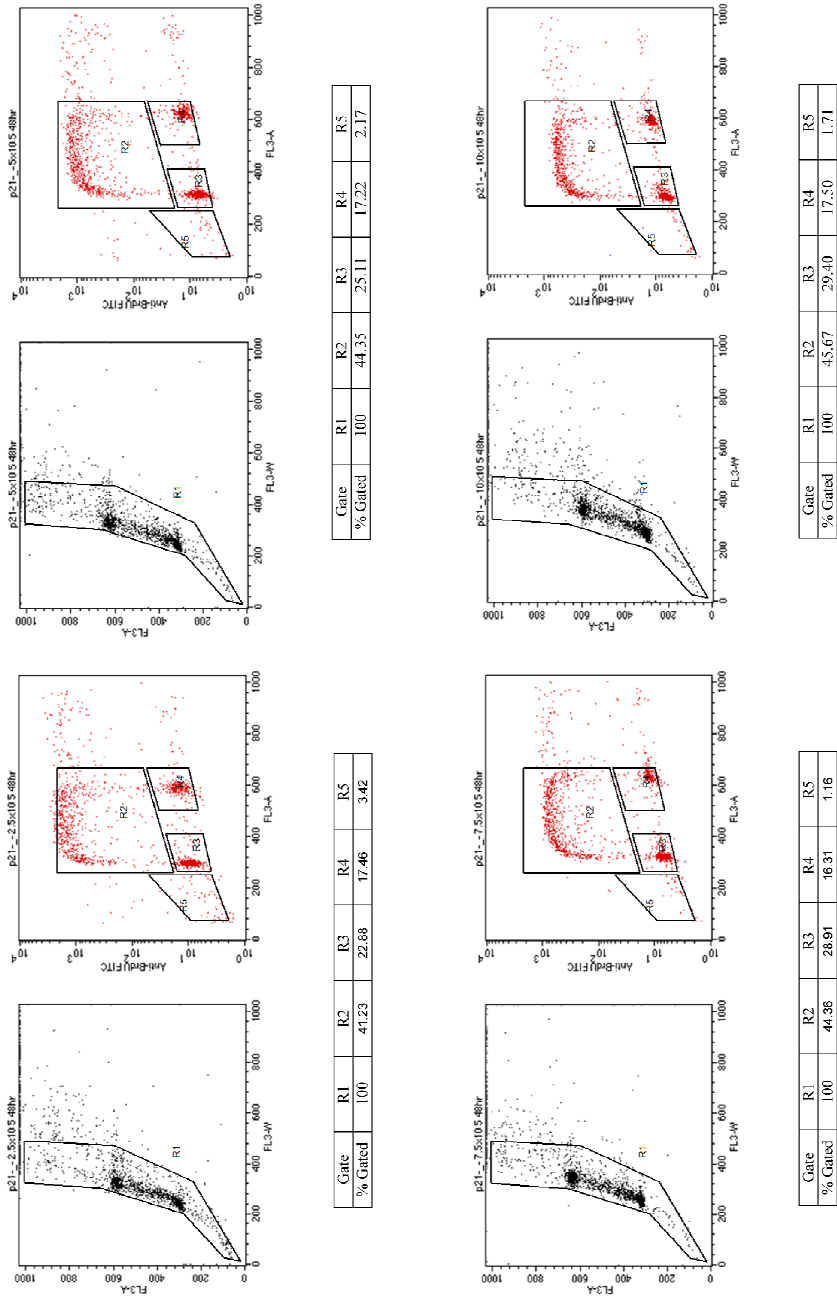


Figure A3: Flow cytometry analysis of HCT116 p21-null cells at increasing seeding densities

HCT116 p21-null cells were seeded at densities of 2.5×10^5 , 5×10^5 , 7.5×10^5 and 1×10^6 and put into the incubator for 48 h. Cells were then harvested, as described in Chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content, and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

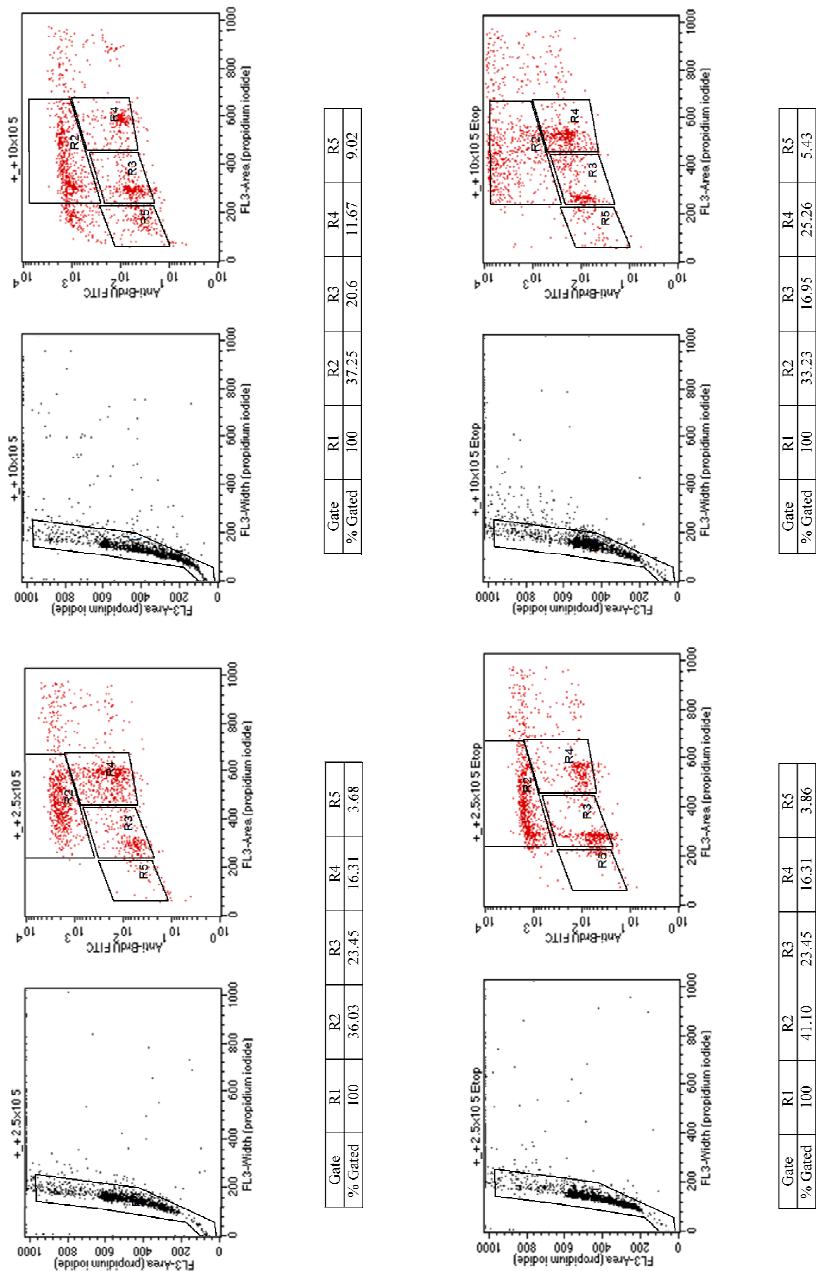


Figure A4: Flow cytometry analysis of HCT116 wild type cells in the absence and presence of Etoposide

HCT116 wild type cells were seeded at densities of 2.5×10^5 and 1×10^6 and put into the incubator for 24 h. Cells were then treated with Etoposide ($100 \mu\text{M}$) and put in the incubator for a further 24 h. Cells were then harvested, as described in Chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content, and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

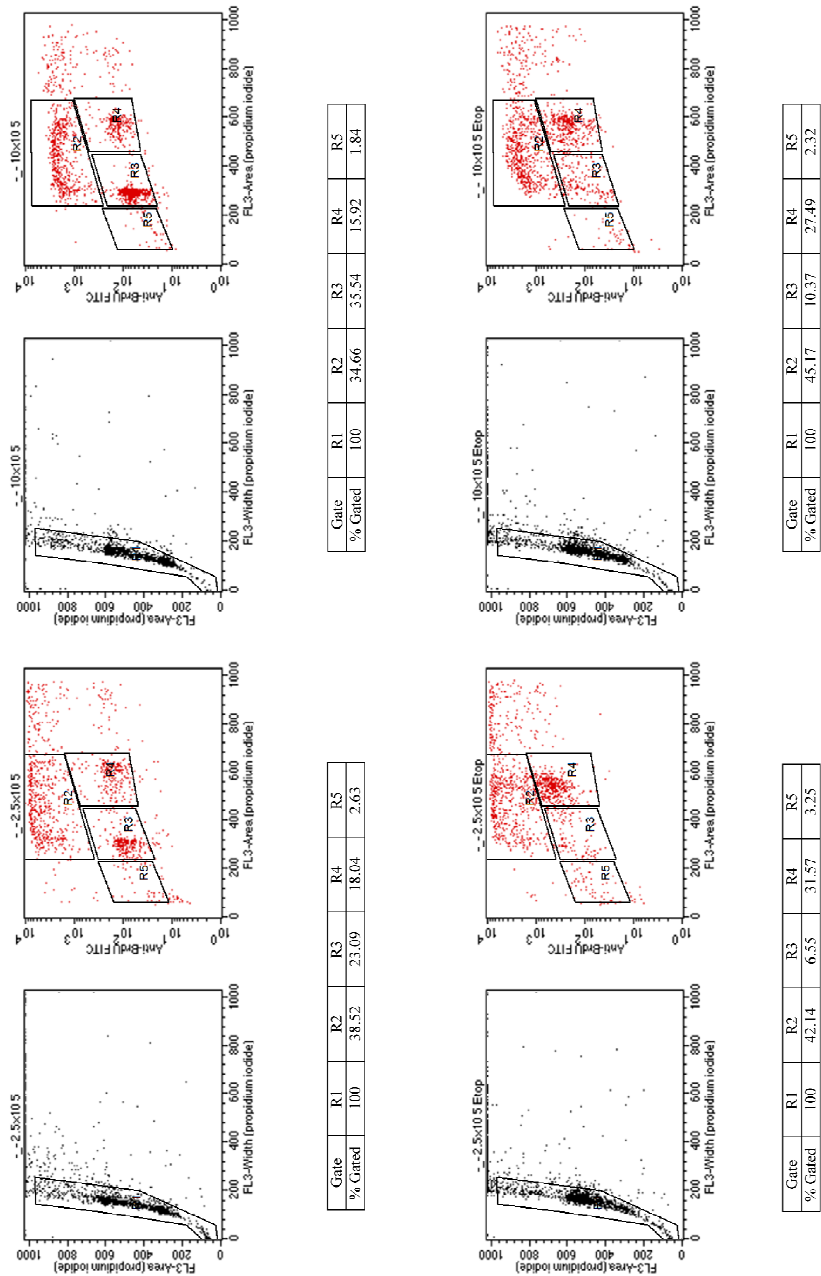


Figure A5: Flow cytometry analysis of HCT116 p53-null cells in the absence and presence of Etoposide

HCT116 p53-null cells were seeded at densities of 2.5×10^5 and 1×10^6 and put into the incubator for 24 h. Cells were then treated with Etoposide (100 μ M) and put in the incubator for a further 24 h. Cells were then harvested, as described in chapter 2, and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

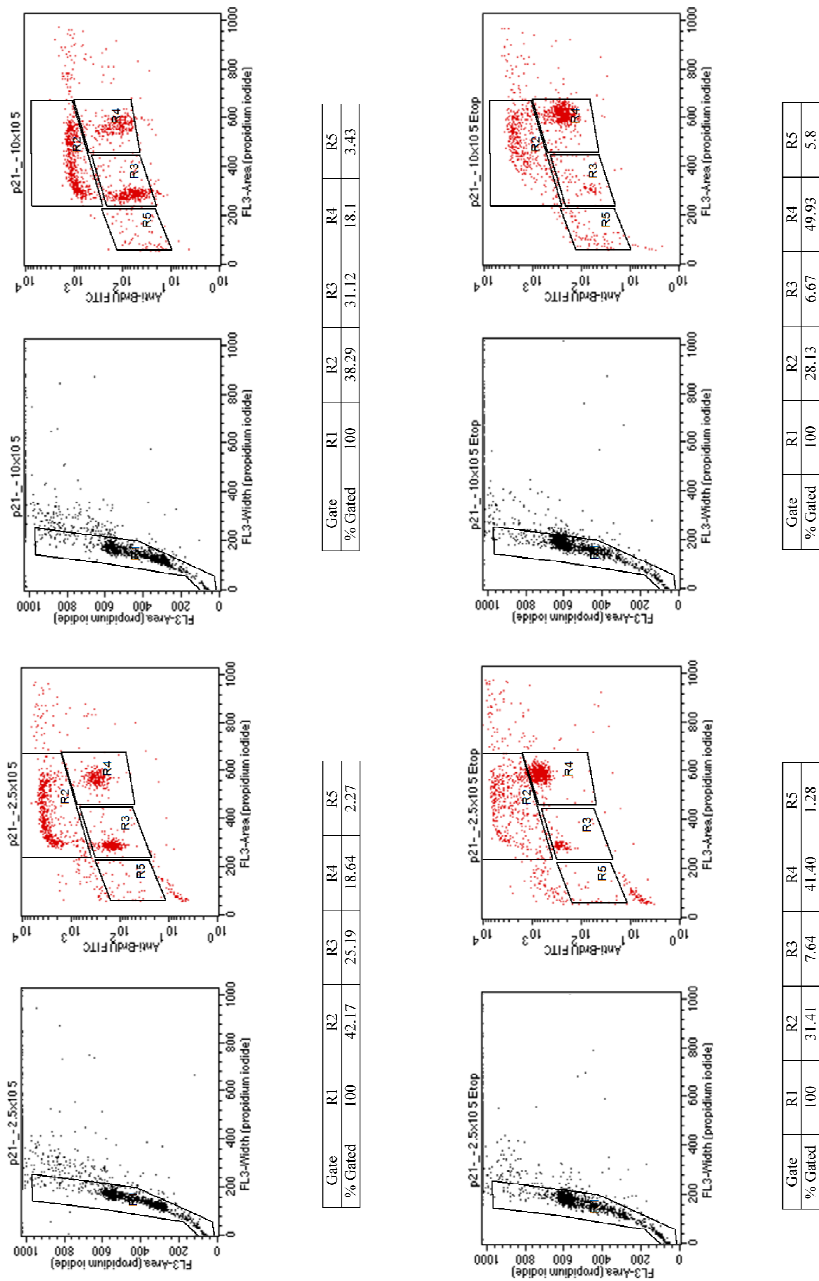


Figure A6: Flow cytometry analysis of HCT116 p21-null cells in the absence and presence of Etoposide

HCT116 p21-null cells were seeded at densities of 2.5×10^5 and 1×10^6 and put into the incubator for 24 h. Cells were then treated with Etoposide (100 μ M) and put in the incubator for a further 24 h. Cells were then harvested, as described in Chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated fusing gate R1 and show the DNA content and stage of cell cycle the cells are in.

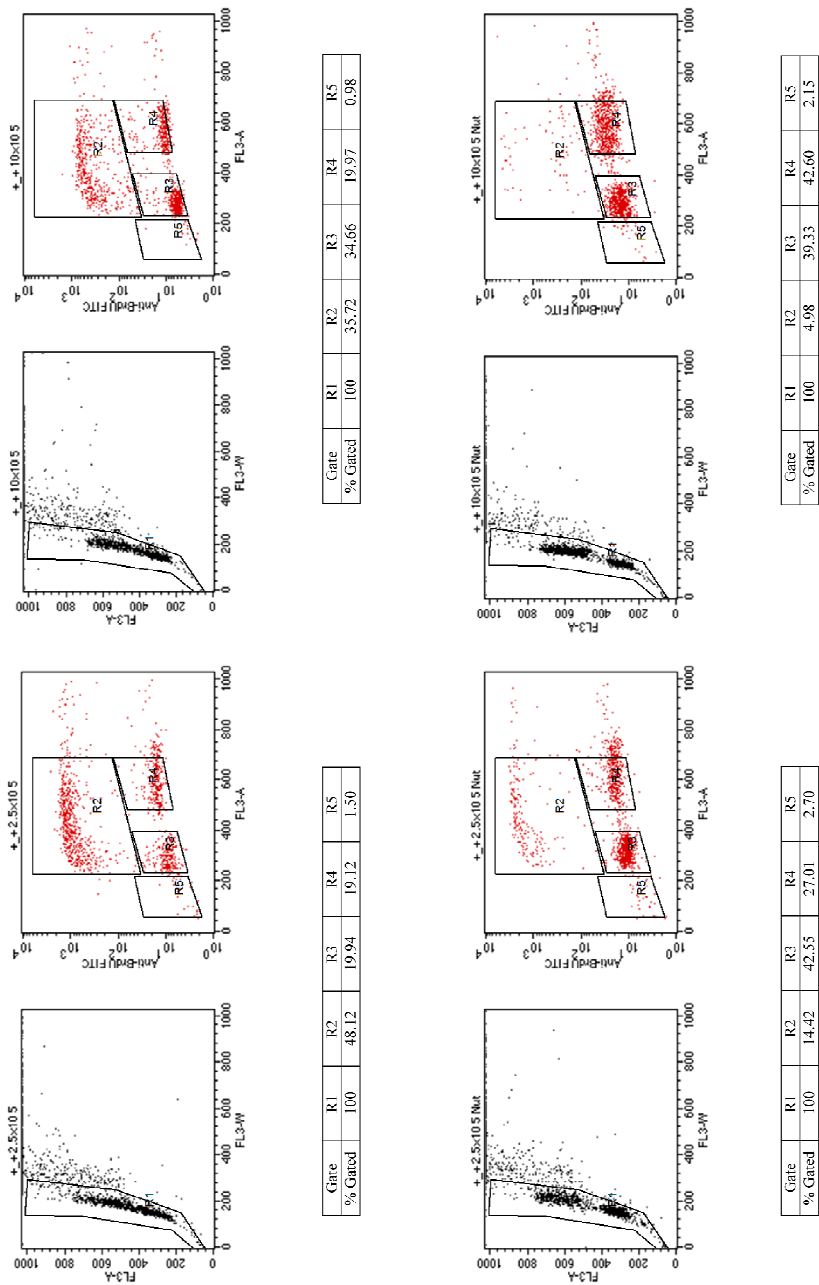


Figure A7: Flow cytometry analysis of HCT116 wild type cells in the absence and presence of Nutlin-3

HCT116 wild type cells were seeded at densities of 2.5×10^5 and 1×10^6 and put into the incubator for 24 h. Cells were then treated with Nutlin-3 (10 μ M) and put in the incubator for a further 24 h. Cells were then harvested, as described in chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content, and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

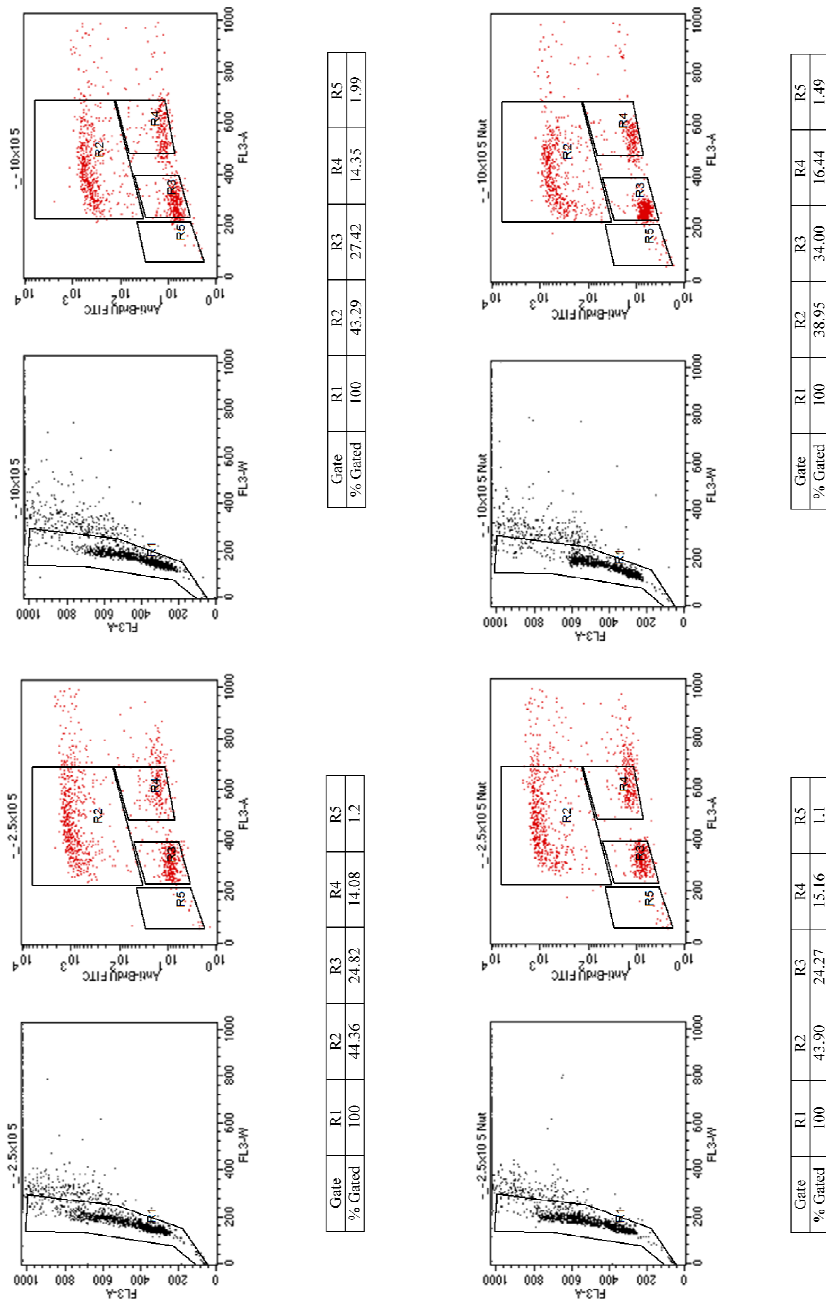


Figure A8: Flow cytometry analysis of HCT116 p53-null cells in the absence and presence of Nutlin-3

HCT116 p53 null cells were seeded at densities of 2.5×10^5 and 1×10^6 and put into the incubator for 24 h. Cells were then treated with Nutlin-3 ($10 \mu\text{M}$) and put in the incubator for a further 24 h. Cells were then harvested, as described in Chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gate R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

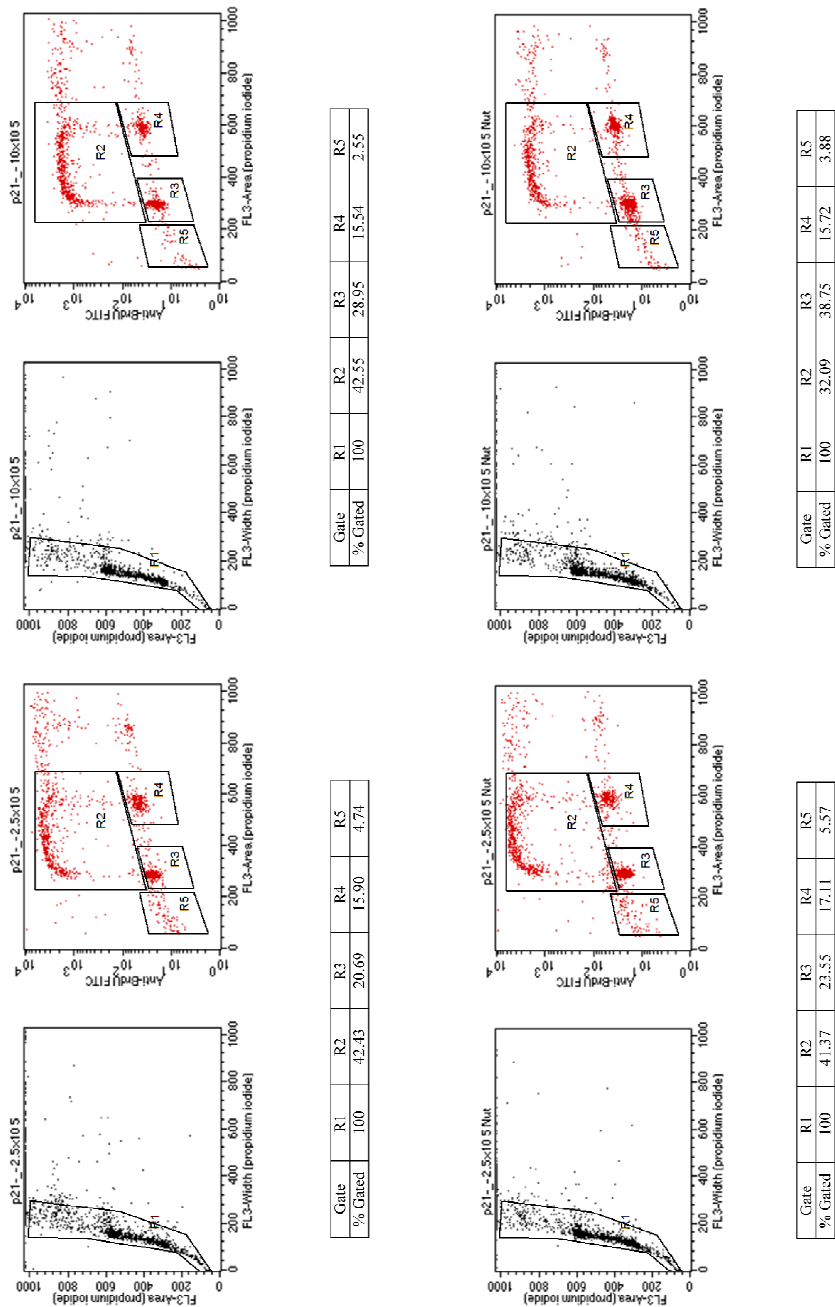


Figure A9: Flow cytometry analysis of HCT116 p21-null cells in the absence and presence of Nutlin-3

HCT116 p21-null cells were seeded at densities of 2.5×10^5 and 1×10^6 and put into the incubator for 24 h. Cells were then treated with Nutlin-3 ($10 \mu\text{M}$) and put in the incubator for a further 24 h. Cells were then harvested, as described in chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

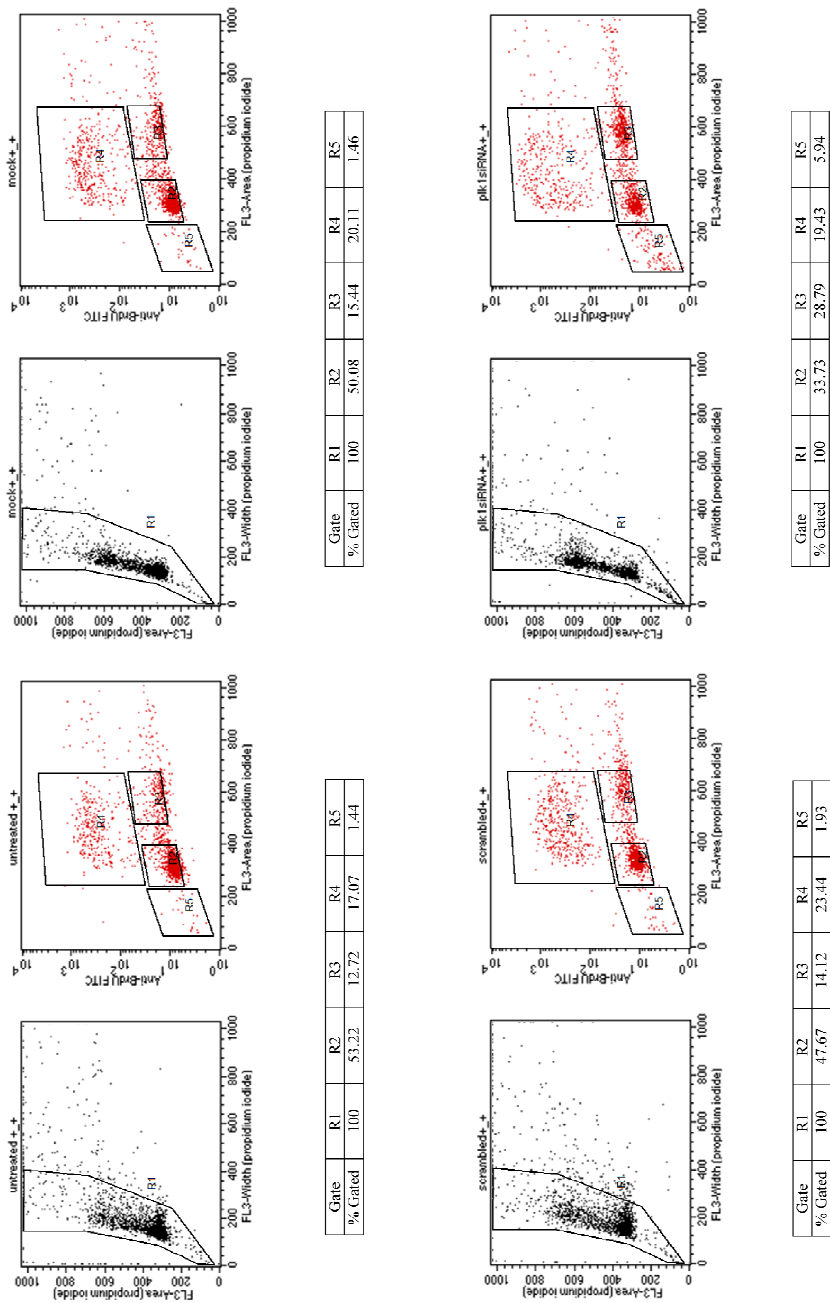


Figure A10: Flow cytometry analysis of HCT116 wild type cells transfected with *PLK1* siRNA

HCT116 wild type cells were seeded at densities of 1×10^6 and put into the incubator for 24 h. Cells were untransfected, mock transfected, transfected with a scrambled siRNA or transfected with *PLK1* siRNA and put in the incubator for a further 48 h. Cells were then harvested, as described in Chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

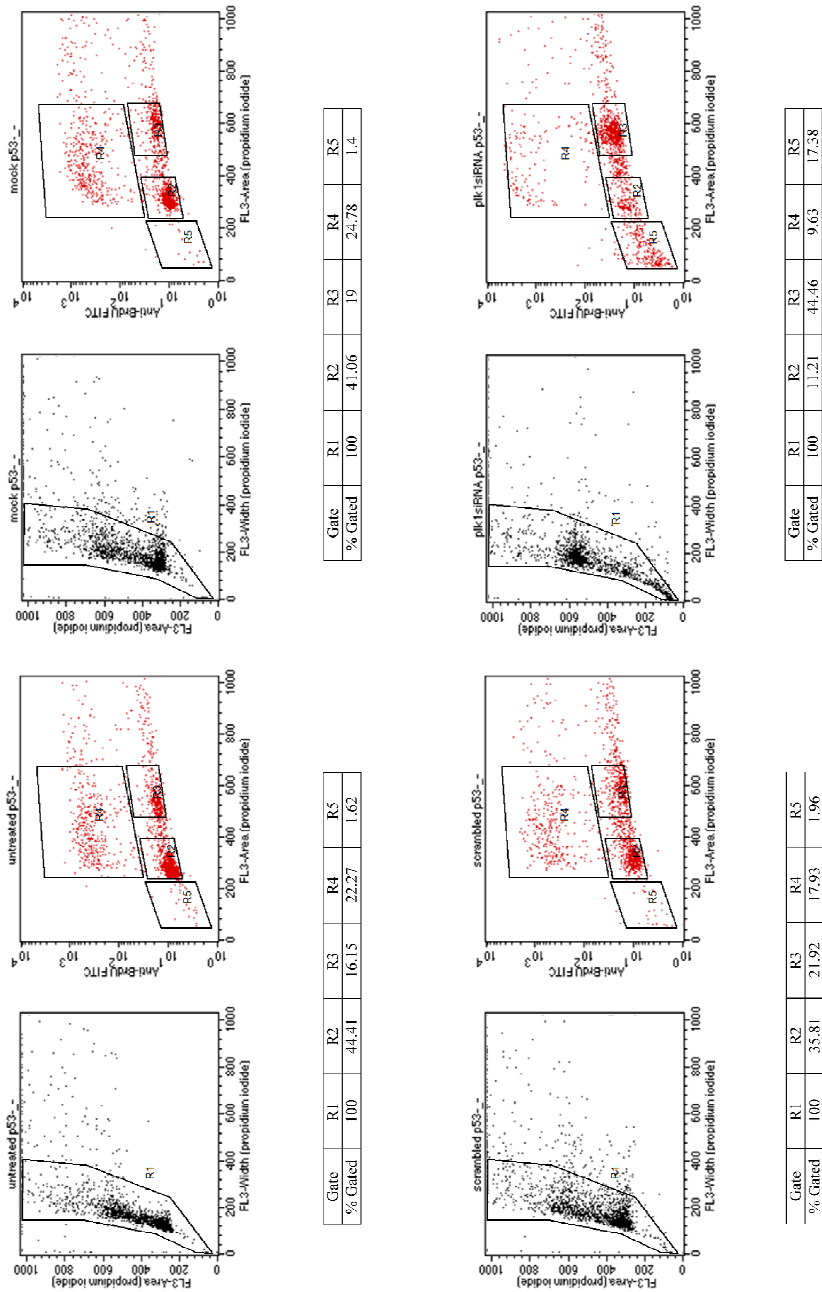


Figure A11: Flow cytometry analysis of HCT116 p53-null cells transfected with *PLK1* siRNA

HCT116 p53-null cells were seeded at densities of 1×10^6 and put into the incubator for 24 h. Cells were untransfected, mock transfected, transfected with a scrambled siRNA or transfected with *PLK1* siRNA and put in the incubator for a further 48 h. Cells were then harvested, as described in Chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

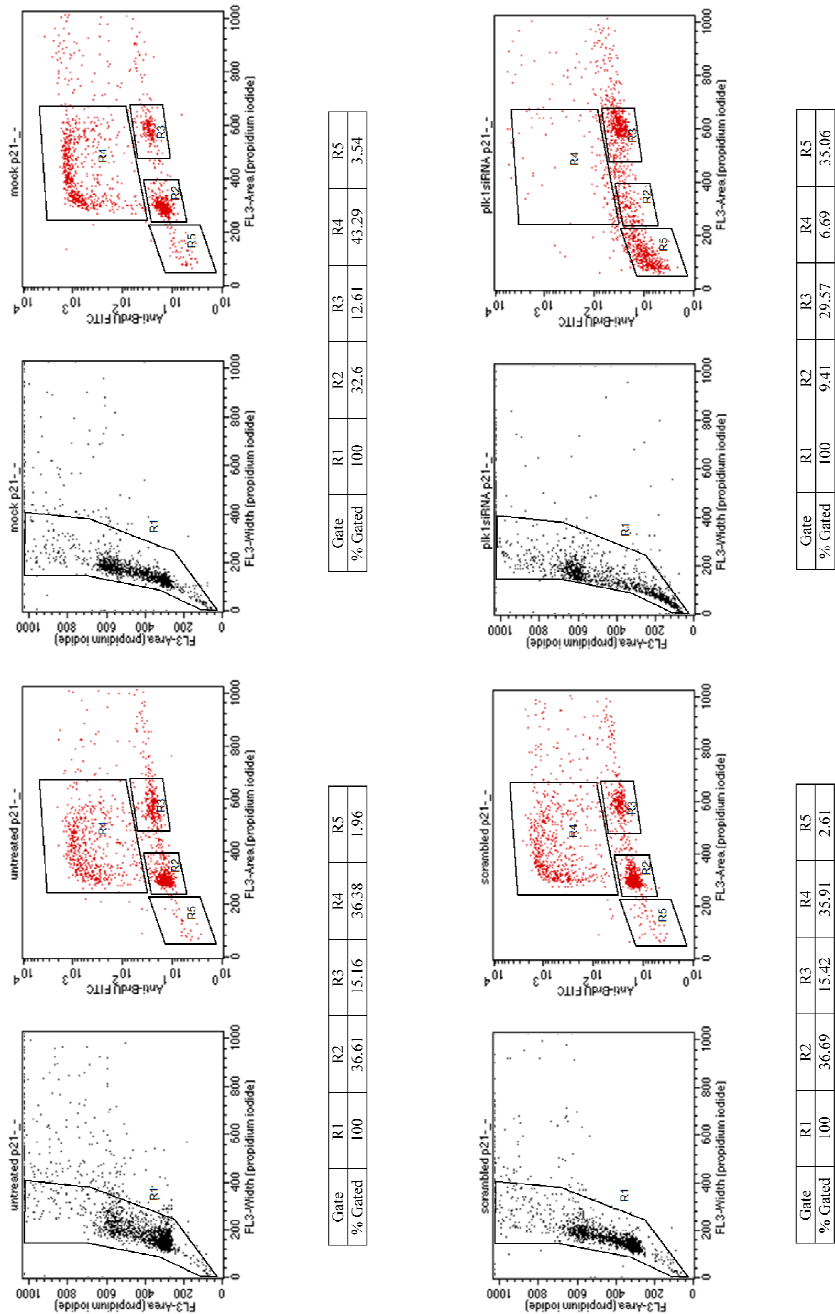


Figure A12: Flow cytometry analysis of HCT116 p21-null cells transfected with *PLK1* siRNA

HCT116 p21-null cells were seeded at densities of 1×10^6 and put into the incubator for 24 h. Cells were untransfected, mock transfected, transfected with a scrambled siRNA or transfected with *PLK1* siRNA and put in the incubator for a further 48 h. Cells were then harvested, as described in Chapter 2 and analysed by flow cytometry. The graphs show the level of propidium iodide intercalating into the DNA, signifying DNA content and the proportion of cells that were undergoing DNA synthesis, signifying the BrdU content, in the cells. Gate R1 shows the overall DNA content of the cells. Gates R2, R3, R4 and R5 are gated using gate R1 and show the DNA content and stage of cell cycle the cells are in.

p53-dependent repression of polo-like kinase-1 (PLK1)

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Key words: p53, repression, G₂/M checkpoint, DNA damage response, PLK1

Abbreviations: ChIP, chromatin immunoprecipitation; PLK1, polo-like kinase-1; p53RE1 and p53RE2, p53 responsive elements-1 and -2 respectively

PLK1 is a critical mediator of G₂/M cell cycle transition that is inactivated and depleted as part of the DNA damage-induced G₂/M checkpoint. Here we show that downregulation of *PLK1* expression occurs through a transcriptional repression mechanism and that p53 is both necessary and sufficient to mediate this effect. Repression of *PLK1* by p53 occurs independently of p21 and of arrest at G₁/S where PLK1 levels are normally repressed in a cell cycle-dependent manner through a CDE/CHR element. Chromatin immunoprecipitation analysis indicates that p53 is present on the *PLK1* promoter at two distinct sites termed p53RE1 and p53RE2. Recruitment of p53 to p53RE2, but not to p53RE1, is stimulated in response to DNA damage and/or p53 activation and is coincident with repression-associated changes in the chromatin. Downregulation of *PLK1* expression by p53 is relieved by the histone deacetylase inhibitor, trichostatin A, and involves recruitment of histone deacetylase to the vicinity of p53RE2, further supporting a transcriptional repression mechanism. Additionally, wild type, but not mutant, p53 represses expression of the *PLK1* promoter when fused upstream of a reporter gene. Silencing of *PLK1* expression by RNAi interferes with cell cycle progression consistent with a role in the p53-mediated checkpoint. These data establish *PLK1* as a direct transcriptional target of p53, independently of p21, that is required for efficient G₂/M arrest.

Introduction

The p53 tumor suppressor is a potent transcription factor that plays a pivotal role in preventing tumor development.¹⁻⁴ p53 is induced in response to a variety of cellular stresses including DNA damage, leading mainly to the onset of apoptosis or cell cycle arrest at the G₁/S and G₂/M boundaries. In addition to activating the expression of a wide range of genes involved in these processes, p53 also represses the expression of many genes required for cell survival and cell cycle progression, including several involved in the G₂/M transition.^{5,6} p53-mediated repression is still only partly understood and its ability to repress G₂/M promoters may involve various mechanisms including interaction with the CCA AT-binding transcription factor NF-Y, direct binding to p53-responsive elements, and/or influence via the CDE/CHR (cell cycle-dependent element/cell cycle genes homology region).⁷⁻⁹

The Polo-Like Kinases are a family of serine/threonine protein kinases that are structurally and functionally related to the prototypic *Drosophila melanogaster* family member, Polo.¹⁰⁻¹³

Mammalian PLK1 plays a pivotal role in the maturation of centrosomes, entry into M phase, spindle formation and cytokinesis.^{10,13} More recently, PLK1 was found to contribute to DNA synthesis where it plays a role in pre-replication complex formation.¹⁴ *PLK1* expression occurs at low levels during the early phase of the cell cycle and is mediated by a repression mechanism involving a promoter element termed CDE/CHR (cell cycle-dependent element/cell cycle gene homology region). However, its levels accumulate throughout S and G₂ phase with a sharp increase in enzymatic activity occurring prior to the onset of M phase.¹⁵⁻¹⁸ PLK1 is oncogenic and constitutive expression of the enzyme causes transformation of NIH3T3 cells.¹⁹ Additionally, PLK1 is upregulated in many human malignancies including colon cancer and is widely considered to be a potential therapeutic target.¹³

p53 plays a critical role in the maintenance of the G₂/M checkpoint⁶ and is protective against DNA damage-induced cell death.²⁰ PLK1 is phosphorylated and inactivated by ATM following DNA damage, leading to arrest at the G₂/M boundary.²¹ Similarly, DNA damage induces downregulation of PLK1 in an

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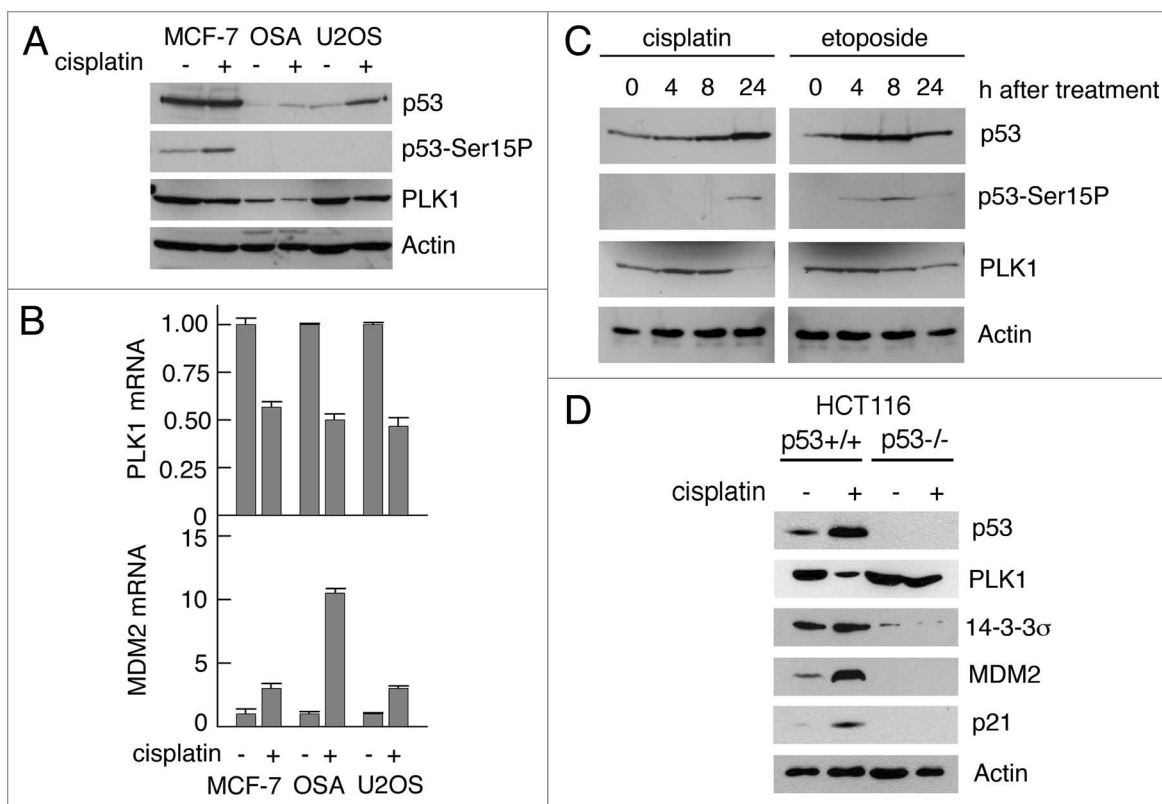


Figure 1. DNA damage downregulates PLK1 protein and mRNA levels in a p53-dependent manner. (A) MCF-7, OSA and U2-OS cells (all expressing wild type p53) were treated with 20 μ M Cis-platin for 24 h. Cells were lysed and western blotting performed with antibodies as indicated. (B) MCF-7, OSA and U2-OS cells were treated as in (A). RNA was extracted and steady-state levels of mRNA encoding PLK1 and MDM2 were determined by real-time quantitative PCR. (C) U2OS cells were treated with 20 μ M cis-platin or -etoposide. Cells were harvested at the indicated times and examined by western blotting. (D) HCT116 p53^{+/+} or p53^{-/-} cells were treated with 20 μ M Cis-platin for 24 h. Cells were lysed and western blotting performed with antibodies as indicated.

ATM/ATR-dependent fashion coincident with increases in p53 and p21.²²⁻²⁴ Several studies have suggested that this is dependent upon p53 and/or p21,²⁵⁻²⁸ but either have not provided any mechanistic insight or have attributed the downregulation to the CDE/CHR element,²⁸ a conclusion that could be explained by a p53-mediated G₁/S arrest. Notably, PLK1 is required for recovery from DNA damage-induced G₂ arrest²⁹ while constitutively active mutants of PLK1 can override the G₂/M checkpoint.²³ These observations underscore the critical contribution of PLK1 activity towards this checkpoint and led us to examine the relationship between PLK1 and the p53 pathway in greater depth.

In the present study we confirm that PLK1 is downregulated following DNA damage. We show conclusively that p53 is both necessary and sufficient to mediate this effect and that it does so through direct repression of *PLK1* expression. We find that p53 is present at two distinct sites in the PLK1 promoter and that its recruitment to one of these is further stimulated by DNA damage in a manner that is coincident with local changes in histone deacetylation favoring a closed chromatin structure. We also eliminate p21-mediated repression through the CDE/CHR element as a major factor in *PLK1* repression. These data are consistent with the idea that PLK1 is quickly suppressed in

a p53-dependent manner as a critical component of the G₂/M checkpoint and have implications for PLK1 levels in tumor cells lacking functional p53.

Results

PLK1 is downregulated in a p53-dependent manner. To determine whether PLK1 levels were regulated in response to DNA damage, three independent cell lines, MCF-7, OSA and U2OS cells (each of which have a wild type p53 response to DNA damage) were treated for 24 hours with the DNA methylating agent, cis-platin. As expected, the drug induced an increase in p53 levels in each cell type (Fig. 1A); a corresponding increase in p53-Ser15 phosphorylation was observed in the MCF-7 cells that have significantly higher basal levels of wild type p53. Notably, in each case, the levels of PLK1 protein were decreased following the treatment with cis-platin (Fig. 1A). Further analysis, by quantitative RT-PCR, indicated that a corresponding decrease in the level of *PLK1* mRNA was observed (Fig. 1B) consistent with the idea the reduced levels of PLK1 protein reflect reduced levels of mRNA expression. In a control analysis, an increase in *MDM2* expression was observed in all three lines confirming that the p53 response to DNA damage was intact in each of these

lines. Treatment of the U2OS cells over a 24 h period with cis-platin or with the DNA strand break-inducer, etoposide, confirmed that PLK1 levels decrease coincidentally with increased p53 levels and activation (Ser15 phosphorylation), and indicate that reduced PLK1 levels occur in response to more than one type of DNA damage-inducing drug (Fig. 1C).

To determine whether the decrease in PLK1 levels was dependent upon the presence of p53, HCT116 cells (HCT116^{+/+}) or an HCT116 derivative lacking expression of full-length p53 (HCT116^{-/-}),³⁰ were treated with cis-platin. While a significant depletion of PLK1 levels was again noted in the p53-competent cells, no changes in PLK1 were evident in the p53-null cells (Fig. 1D). These data indicate that cis-platin-induced PLK1 depletion is dependent upon wild type p53. Control analyses showed that increased expression of 14-3-3 σ , MDM2 and p21 occurred only in the p53-competent cells confirming that the p53 pathway was functional only in the HCT116^{+/+} cells (Fig. 1D).

p53 is necessary and sufficient to mediate decreased PLK1 levels. To explore further the contribution of the p53 pathway towards downregulation of PLK1 HCT116 cells were treated with increasing amounts of Nutlin-3 which blocks the interaction of p53 with its binding site in the N-terminus of MDM2.³¹ Following this treatment, a dose-dependent increase in p53 levels was accompanied by matching increases in the levels of p21, 14-3-3 σ and MDM2 (Fig. 2A). Notably, a dose dependent decrease in PLK1 levels was also observed. These changes were apparent only in the HCT116^{+/+} cells and not in the HCT116^{-/-} cells, again consistent with a critical role for p53 in PLK1 reduction. Additionally, treatment of the HCT116^{+/+} cells, but not the HCT116^{-/-} cells, with Nutlin-3 resulted in a dose-dependent decrease in *PLK1* mRNA (Fig. 2B). Furthermore, a time course analysis showed that the reduction in PLK1 levels was co-incident with the accumulation of p53 and p21 (Fig. 2C). Strictly speaking, Nutlin-3 is an MDM2 inhibitor, raising the possibility that the changes in PLK1 levels could arise as a result of inhibition of a p53-independent function of MDM2. To address this issue, the HCT116^{-/-} cells, in which

endogenous MDM2 levels are barely detectable, were infected with adenoviruses expressing wild type MDM2 or, as a control, GFP. Treatment of the cells with Nutlin-3, in the absence or presence of MDM2, had no effect on PLK1 levels ruling out this trivial explanation (Fig. 2D). (The MDM2 levels were slightly higher in the presence of Nutlin-3 consistent with the idea that the drug may inhibit the ability of MDM2 to mediate its own destruction). While the data favor a mechanism whereby p53 can

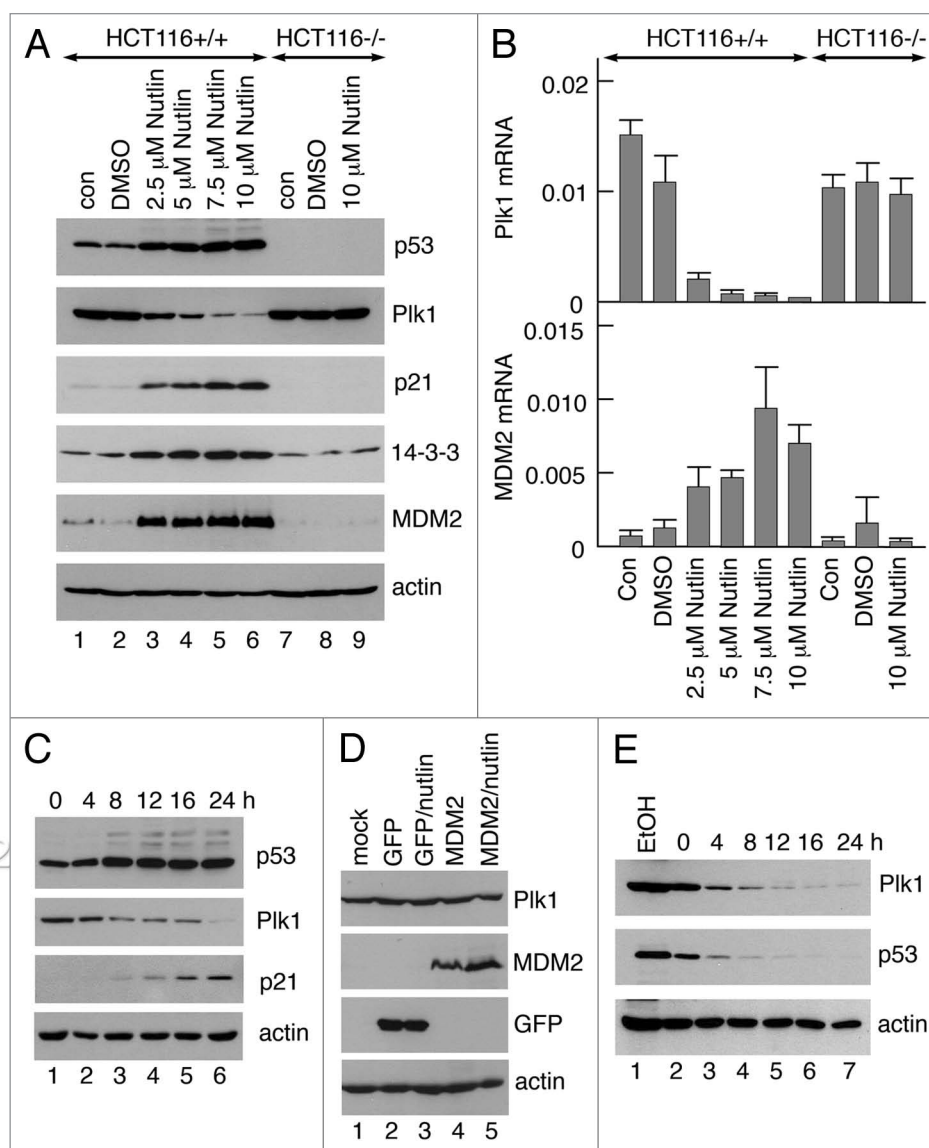


Figure 2. Nutlin-3 induces p53-mediated downregulation of PLK1. (A) HCT116 p53^{+/+} or p53^{-/-} cells were treated with 0–10 μ M Nutlin-3 or DMSO as vehicle control. The cells were lysed and western blotting was performed with the indicated antibodies. (B) HCT116 cells were treated as in (A) and the steady-state levels of mRNA encoding PLK1 and MDM2 were determined by real-time quantitative PCR. (C) HCT116 p53^{+/+} cells were treated with 5 μ M Nutlin-3 for 0–24 h. Cells were lysed and western blotting performed with the antibodies as indicated. (D) HCT116 p53^{-/-} cells were infected with recombinant viruses Ad-MDM2 or Ad-GFP at an MOI of 50 for 4 h. The cells were then treated with 10 μ M Nutlin-3 for 24 h and lysed before western blotting was performed with the indicated antibodies. (E) HCT116 p53 wild-type cells were treated with 10 μ g/ml Cycloheximide and cell lysates prepared at the indicated time points after addition. western blotting was then performed with the antibodies as indicated.

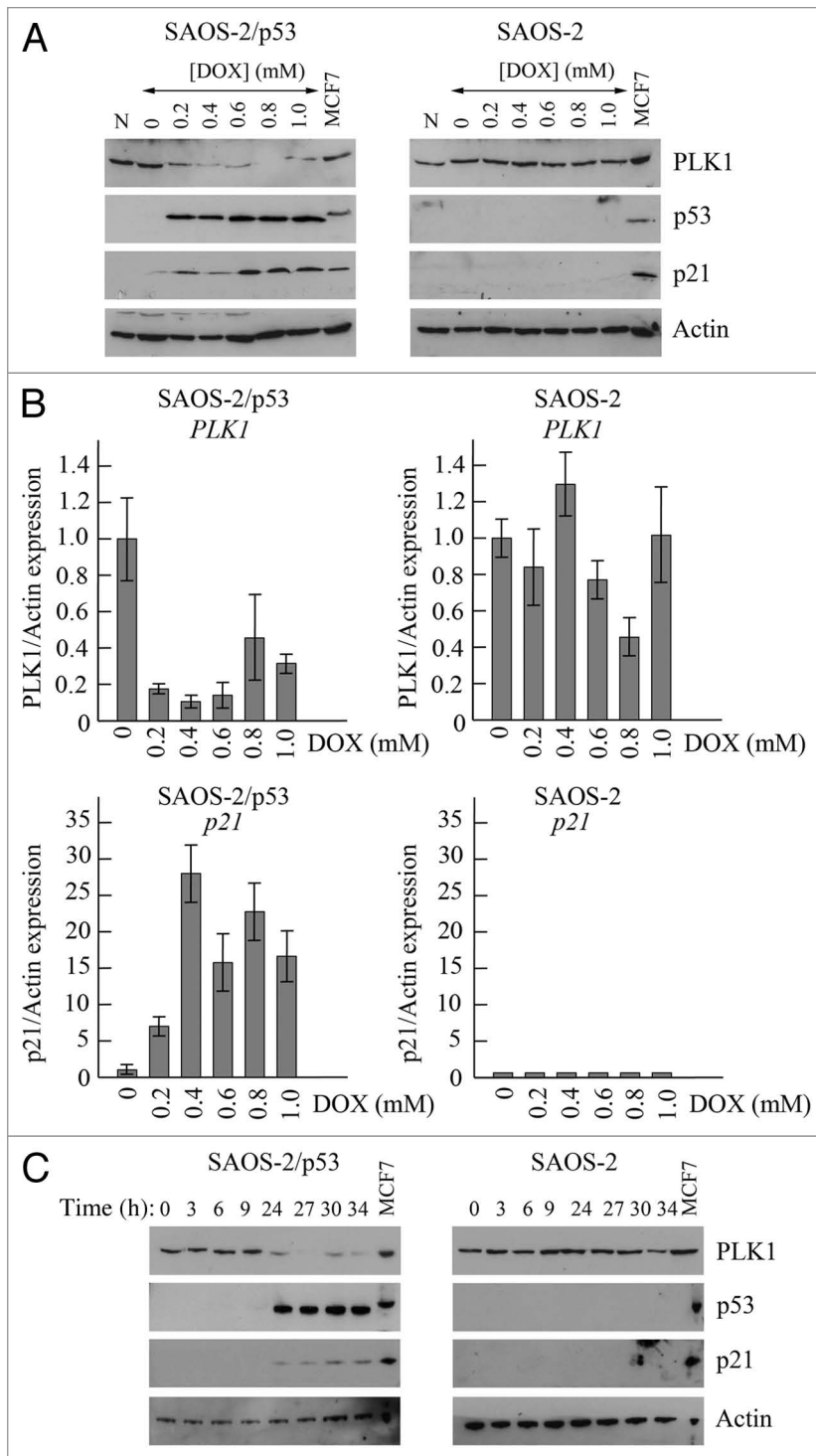


Figure 3. Downregulation of PLK1 levels in SAOS-2 cells following inducible ectopic expression of p53. SAOS-2 cells and a derivative line that ectopically expresses mouse p53 in a doxycycline-inducible manner³² were treated for 24 h in the presence of increasing amounts of doxycycline. After harvesting, the cell extracts were analysed by western blotting (A) or quantitative RT-PCR (B) to determine the levels of PLK1, p53, p21 and, as control, actin. The cells were also treated with 0.5 mM doxycycline for up to 34 h prior to harvesting. The levels of PLK1, p53, p21 and actin were subsequently determined by western blotting (C).

repress *PLK1* expression, it is also possible that p53 may promote the turnover of PLK1 protein. However, analysis of the decay of PLK1 protein in the absence of Nutlin-3 indicated that its half life (determined by cycloheximide-chase) was not significantly different when compared with the decay rate following treatment of the cells with Nutlin-3 (compare Fig. 2E and C). It is already established that PLK1 is degraded through the ubiquitin-proteasome pathway, most likely mediated by APC (reviewed in ref. 10).

PLK1 levels have been reported to be low during the early stages of the cell cycle and accumulate throughout S and G₂ phases.¹⁵⁻¹⁸ These published observations were verified in the present study (Suppl. Fig. 1). This effect is mediated through a repressor element, termed CDE/CHR, that operates during the early phase of the cell cycle.¹⁸ The reduced level of PLK1 protein and -mRNA could therefore simply reflect the accumulation of cells at G₁/S following a p53-dependent cell cycle arrest. To explore this possibility, SAOS-2 cells were used together with a derivative line (SAOS-2/p53,³²) in which wild type mouse p53 is inducibly expressed using a doxycycline-inducible promoter. (SAOS-2 cells, which lack endogenous p53, also lack Rb and cannot therefore arrest at G₁/S). Upon treatment of the SAOS-2/p53 cells with doxycycline, p53 was induced in a dose-dependent (Fig. 3A) and time-dependent (Fig. 3C) manner leading to increases in the levels of p21 protein and—mRNA; (p21 is both a p53 target and is primarily responsible for mediating p53-dependent G₁/S arrest). Significantly, the levels of PLK1 protein (Fig. 3A and C) and mRNA (Fig. 3B) were depleted following induction of p53. These changes were not observed following doxycycline treatment of the parental SAOS-2 cells that lack the inducible p53. These data indicate that downregulation of PLK1 expression can be achieved in the absence of a p53-dependent G₁/S arrest.

Using a parallel approach, the expression of p21 was silenced in two independent cell lines using siRNA (Fig. 4A). Following treatment of the cells with Nutlin-3, significant downregulation of PLK1 levels were still observed even though there was no detectable p21 in the silenced cells. It should be noted, however, that the downregulation of PLK1 by Nutlin-3 was slightly less efficient in the p21-silenced cells as compared with the control treatments (especially in the HCT116 cells) suggesting that p21 is likely to make a minor contribution to this effect. To confirm this result, p21 expression was silenced in SAOS-2/p53 cells. Again, downregulation of *PLK1* expression was independent of the presence of p21 (Fig. 4B).

PLK1 levels were also measured in HCT116^{+/+} cells null for p21³⁰ as compared with the HCT116^{+/+} and HCT116 p53^{-/-} cells following induction of p53 (Suppl. Fig. 2). The data indicate that downregulation of PLK1 levels by all three stresses (etoposide, Nutlin-3 and cis-platin) is partially impaired in the absence of p21, particularly at the time point measured (24 h post-treatment: see A). Nutlin-3, which is the most potent of these treatments in terms of inducing the p53 response, still retains significant ability to downregulate PLK1 levels. Notably, however, etoposide was also able to downregulate PLK1 levels in the p21-null cells at earlier time points although less robustly in comparison with the parental cells, and concomitantly with a much reduced induction of p53 (as measured by the increase in p53 levels and the induction of p53-responsive genes such as MDM2 and 14-3-3σ [Suppl. Fig. 2A and C], and with a partial recovery by 24 h). Flow cytometry confirmed that Nutlin-3 induces both a G₁/S and G₂/M block in the wild type cells but cell cycle arrest is abolished in the cells lacking p53 or p21 (Suppl. Fig. 2B). This observation suggests that the p53-dependent decrease in PLK1 levels cannot simply be attributed to an arrest at G₁/S where the PLK1 levels are naturally low.

p53 binds directly to the PLK1 promoter and inhibits PLK1 transcription. The downregulation of PLK1 at both the protein and mRNA levels is consistent with the idea that p53 can mediate transcriptional repression of *PLK1*. If such a mechanism occurs then it should be possible to detect the presence and/or recruitment of p53 to the *PLK1* promoter. To test this idea, chromatin immunoprecipitation (ChIP) experiments were carried out. Two potential p53 binding sites were observed in the *PLK1* promoter at positions -2,067 to -2,016 (termed p53 responsive element 1 [p53RE1]) and -816 to -785 (p53RE2; Fig. 5A): the latter site contains two contiguous canonical p53 half-sites.⁵ Accordingly, PCR primers were designed to permit analysis of each of these two regions. Additionally, given that p53 can repress promoters via interactions with factors operating within core promoter elements (for example through interaction with the CCAAT box-associated NF-Y transcription factor⁷ or the CDE/CHR elements²⁰) PCR primers were designed to permit analysis of the core promoter region. ChIP analysis of the *PLK1* promoter revealed that p53 was present within both of the regions encompassing putative p53 consensus binding sites (p53RE1 and p53RE2 respectively: Fig. 5B and C, left hand parts) but could not, however, be detected within the core promoter region containing the CAATT and CDE/CHR elements (Fig. 5D). As control, no p53 was detected on the *GAPDH* promoter (Fig. 5E). Interestingly, when p53 was induced either through treatment with etoposide or Nutlin-3 a modest increase in the levels of recruitment of p53 were observed at the region of the *PLK1* promoter containing p53RE2, but not that containing p53RE1. This was accompanied by a striking decrease in the level of acetylated histone H3 present at p53RE2 (Fig. 5C, right hand side), consistent with structural changes in the chromatin normally associated with

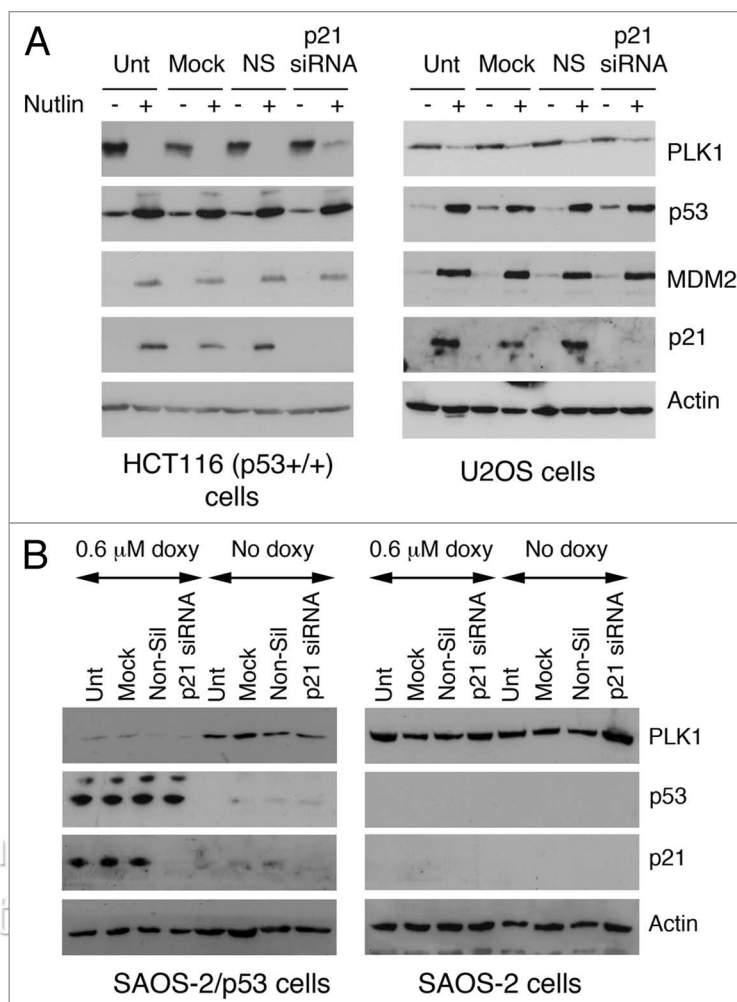


Figure 4. Downregulation of PLK1 can occur following elimination of p21. (A) Expression of p21 in HCT116 cells or U2OS cells was silenced by siRNA. Control cells were untreated (Unt), mock-transfected (Mock), or transfected with a non-silencing siRNA. Cells were subsequently treated with 5 μM Nutlin-3 for 24 h prior to harvesting. Western blotting was subsequently performed with the antibodies as indicated. (B) Expression of p21 in SAOS-2 cells expressing p53 under an IPTG-inducible system (or parental cells lacking p53, as control) was silenced by siRNA treatment as in (A). Western blotting was subsequently performed with the antibodies as indicated.

transcriptional repression. Acetylated H3 was also observed in the vicinity of p53RE1 but was unaltered following stimulation of p53, indicating that the interaction of p53 with this region of the promoter did not mediate repression-associated chromatin remodeling.

To confirm that *PLK1* expression is downregulated by a p53-dependent repression mechanism, HCT116 parental cells and the HCT116-p53^{-/-} derivatives were treated with the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA). Treatment of the cells with TSA alone led to a reduction in PLK1 levels independently of p53 (Fig. 6A): this observation probably reflects the widespread involvement of HDACs in regulating gene expression; (its p53-independent effect on *PLK1* expression is most plausibly explained if TSA relieves expression of a *PLK1* negative regulator). However, treatment of the parental cells, but not the

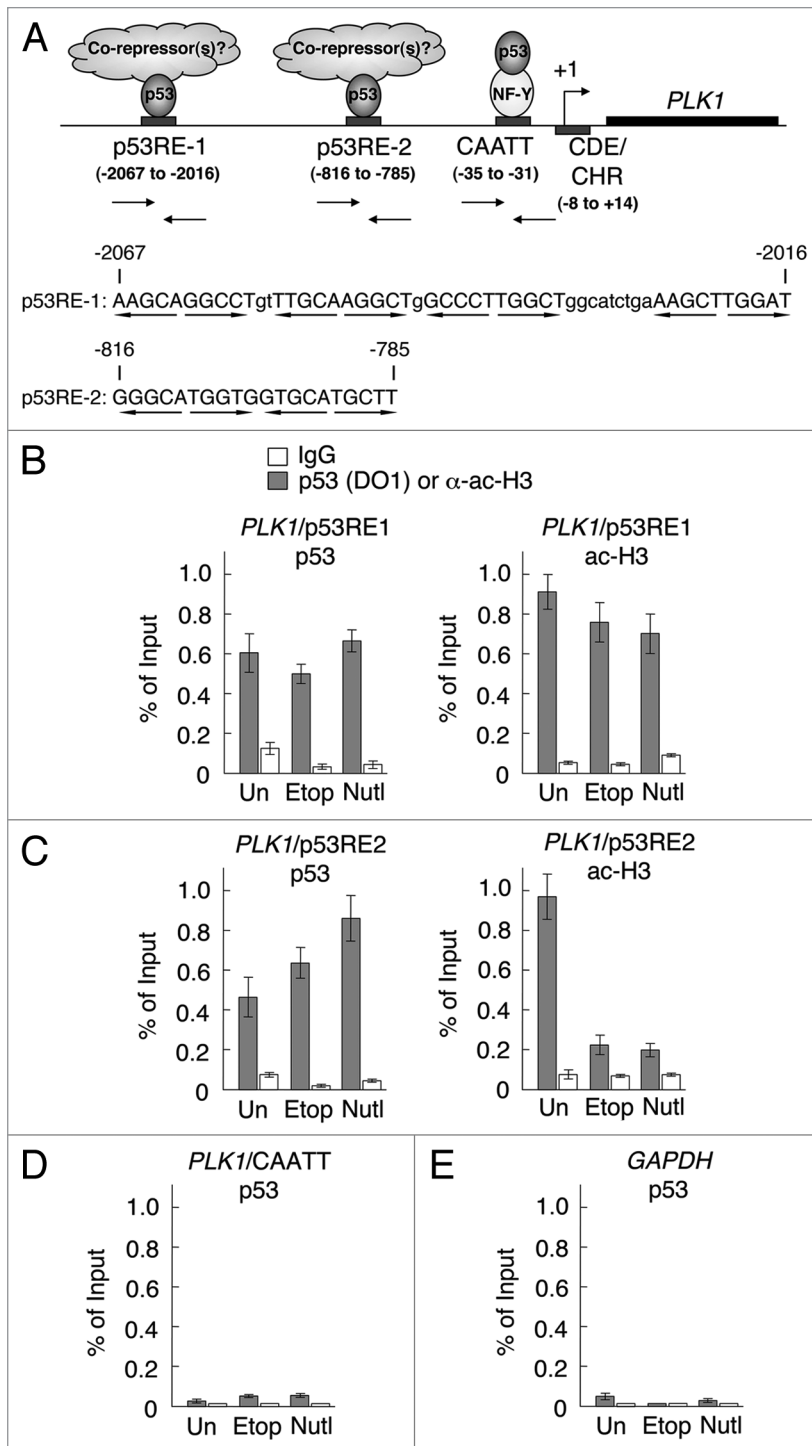


Figure 5. p53 is recruited to the *PLK1* promoter in response to DNA damage. (A) Schematic showing the *PLK1* promoter. The positions of the two putative p53 responsive elements (p53RE1 and p53RE2) are shown together with the CAATT box and the CDE/CHR element. The numbers in parentheses indicate the positions of these elements relative to the transcriptional start site. The arrows show the positions of primers/probes designed to examine these regions by quantitative ChIP. The sequences of the regions containing potential p53 responsive elements p53RE1 and p53RE2 are shown beneath and the bases within these elements are shown in upper case. Quarter sites are indicated by arrows. The sequence information is taken from Genbank sequence X90725. (B–E) HCT116 cells were treated with 50 μ M etoposide (Etop), 10 μ M Nutlin-3 (Nutm) or were untreated (Un) as control. Cells were harvested after 8 h and the presence of p53 and acetylated histone H3 on the p53RE1 (B) and p53RE2 (C) regions of the *PLK1* promoter were determined by ChIP. Control immunoprecipitations were carried out using IgG. ChIP analyses was extended to the core promoter region where the CAATT box and CDE/CHR elements are located (D). As a negative control, the GAPDH promoter was examined for the presence of p53 (E). The measurements were carried out in triplicate and the results are representative of three independent experiments.

presence of HDAC1 is enriched within the vicinity of p53RE2 in response to etoposide treatment consistent with the idea that HDAC1 is recruited to the promoter as part of the repression mechanism. In contrast, the presence of HDAC1 on the p53-stimulated *p21* promoter was reduced under these conditions, as might be expected with activation of this promoter (Fig. 6B).

To determine whether p53 can bind specifically to the *PLK1* promoter, EMSA experiments were carried out using complementary DNA oligonucleotides representing p53RE2 (Fig. 7: p53RE2 was examined because it is responsive to stimuli that activate p53 [see above] and is located in the vicinity of the repression-associated histone deacetylation events). Bacterially-expressed p53 bound to the p53RE2 promoter element and its binding was both shifted and stimulated by the anti-p53 C-terminal antibody, PAb421. (The p53 protein has both a site-specific binding function [encompassed within the core domain of the protein] and a non-specific DNA binding function [located at

p53^{-/-} cells, with Nutlin-3 alone led to a significant downregulation of PLK1 levels over and above that seen with TSA alone. This inhibition was relieved in the presence of TSA where the PLK1 levels recovered to the level of PLK1 in the cells that had been treated with TSA alone. These data support the idea that p53-mediated downregulation of *PLK1* expression occurs via HDAC-mediated transcriptional repression. To provide a more focussed examination of the involvement of histone deacetylase, quantitative ChIP analysis was carried out using an antibody that recognizes HDAC1 (Fig. 6B). The data indicate that the

the C-terminus]: the PAb421 antibody is an established activator of the site-specific-, but not the non-specific-DNA binding function of p53).³³ p53 was still able to interact with a mutant of the p53RE2 response element in which three base pairs were changed. However, this binding, although super-shifted by PAb421, was not stimulated by the antibody suggesting that it occurred through the non-specific binding function of p53. By comparison, similar differences in site-specific and non-specific binding are illustrated in the controls for p53/DNA binding where the appropriate sequences used by Hupp and colleagues

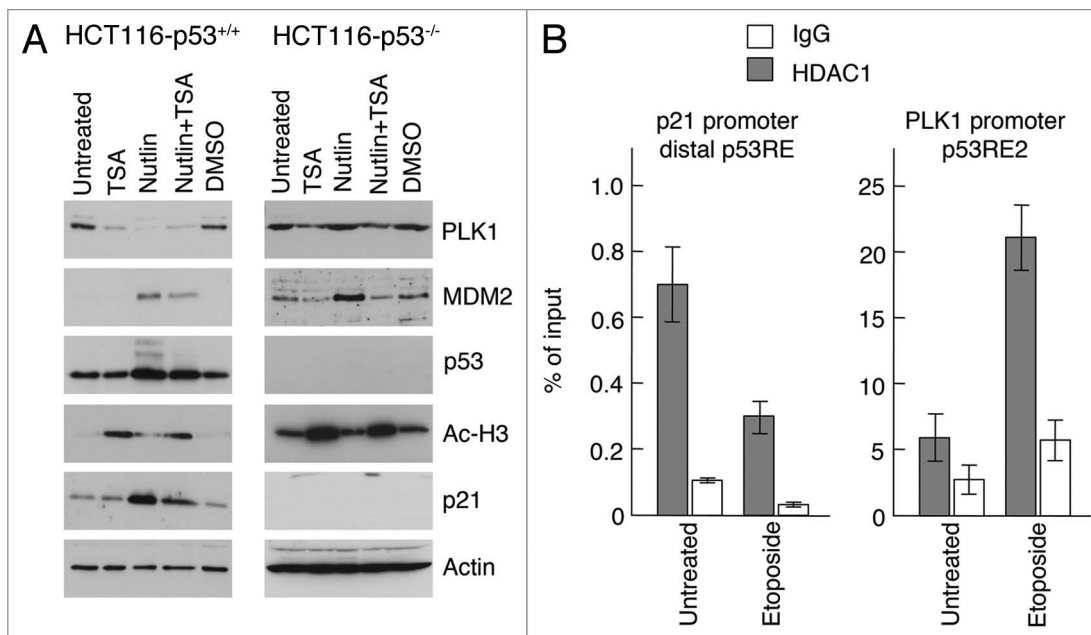


Figure 6. Histone deacetylase activity contributes to downregulation of PLK1 by p53. (A) HCT116 parental cells or the p53-null derivative were treated with 1 μ M trichostatin A (TSA) alone, 10 μ M Nutlin-3, or a combination of these two drugs. Cells were lysed after 24 h and western blotting performed with antibodies as indicated. (B) HCT116 cells were treated for 8 h with 50 μ M etoposide. Chromatin immunoprecipitation analysis was carried out using an antibody that recognizes HDAC1.

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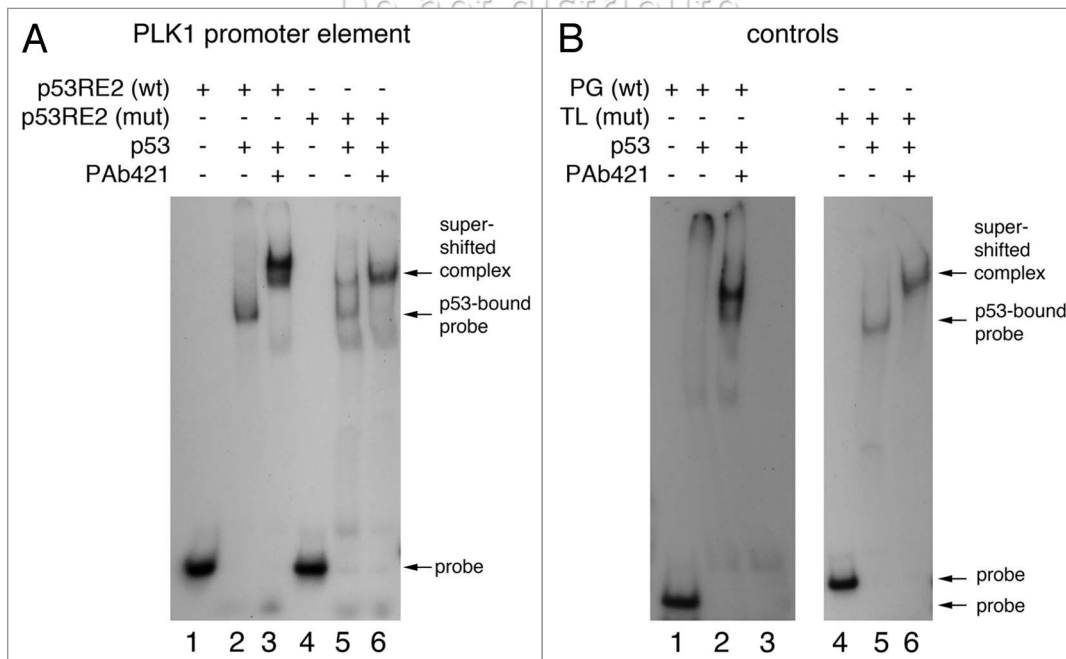


Figure 7. p53 binds directly to the p53RE2 sequence. (A) EMSA was carried out using radiolabeled dsDNA probes corresponding to the p53RE2 sequence (lanes 1–3) and a mutant derivative (lanes 4–6) in which three base pairs were substituted to destroy the p53 binding site consensus: (the sequence GTG CAT GCT T was changed to GTG gAT tgT T [with the changed bases shown in lower case]). (B) EMSA showing the binding of p53 to the control sequences PG (comprising a canonical p53 responsive element, lanes 1–3) and TL (comprising a sequence unrelated to a canonical p53 binding site, lanes 4–6). These reagents are established tools with which to measure site-specific p53 binding.³³

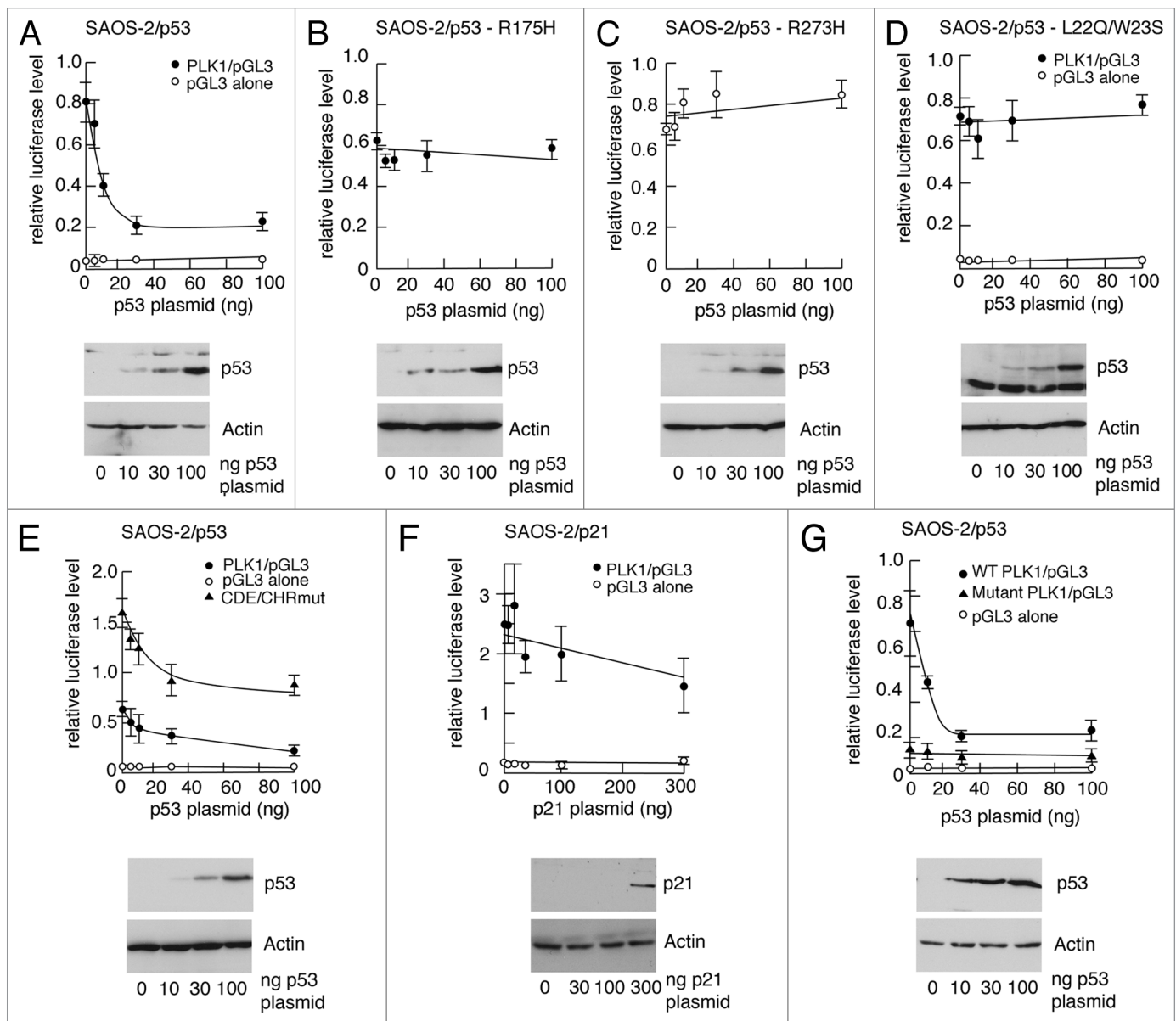


Figure 8. p53 represses expression of the *PLK1* promoter. SAOS-2 cells were transfected in triplicate with increasing amounts a plasmids encoding wild type or mutant p53 or p21, together with promoter/(Firefly) luciferase vectors containing a wild type or mutated *PLK1* promoter and, as an internal standard, a plasmid expressing *Renilla luciferase*. Cell extracts were made 24 h after transfection and the levels of luciferase were determined. The graphs show the ratios of firefly luciferase/*Renilla luciferase* with error bars representing the standard deviations. Western blots shown under each graph show representative increases in the levels of expression of p53 or p21 with actin as loading control.

were employed.³³ These data support the idea that p53RE2 constitutes an effective p53 binding site.

p53 represses the expression of the *PLK1* promoter in a promoter-reporter assay. To determine whether p53 can directly repress expression from the *PLK1* promoter, 2.4 kb of the promoter was cloned upstream of a luciferase reporter gene (Fig. 8). The promoter-luciferase plasmid was transfected into SAOS-2 cells (Fig. 8) or HCT116-p53^{-/-} cells (Suppl. Fig. 3) together with increasing amounts of a plasmid expressing wild type human p53. These analyses showed that very low levels of p53 (low nanogram amounts of plasmid) inhibit expression (Fig. 8A; similar levels of p53 are required to stimulate the expression from the *p21*

promoter [data not shown]). Interestingly, repression mediated by increasing amounts of p53 reached a threshold (with generally 20–40% of the activity remaining) beyond which further increases in the amount of p53 had no further effect. Two tumor-associated inactive mutants of p53 that cannot bind DNA failed to repress the *PLK1* promoter (Fig. 8B and C). Additionally the L22Q/W23S mutant of p53 (Fig. 8D), which is inactive in transactivation,³⁴ was also unable to repress the *PLK1* promoter suggesting that this domain participates in the repression mechanism. (Others have established previously that this region of p53 also contributes to transcriptional repression).³⁵ Mutation of the CDE/CHR element did not eliminate repression by p53 (Fig.

8E) indicating that p53-mediated repression occurs independently of this important repressor element: however, the basal levels of expression from the *PLK1* promoter increased by 2–3-fold consistent with published data indicating that this element mediates cell cycle-dependent repression of *PLK1*.¹⁸ Titration of a plasmid encoding active p21 (which was able to arrest cells at G₁/S [data not shown]) had no significant effect on expression from the *PLK1* promoter although the trend over several experiments suggested the possibility of a small contribution by p21 (Fig. 8F).

To determine whether the repression was mediated through p53RE2, the same three mutations that were present in the mutant p53RE2 element used in the EMSA experiments (Fig. 7) were introduced into the *PLK1* promoter-luciferase plasmid. *PLK1* promoter-luciferase analysis with this mutant promoter indicated that the p53 responsiveness does indeed appear to be diminished (Fig. 8G). Additionally, however, there was a striking reduction in the basal level of expression suggesting that one or more of the bases we had changed are vital to maintaining basal *PLK1* expression (Fig. 8G). This observation suggests the possibility that the binding site for a stimulatory factor may overlap with the p53 binding site.

PLK1 depletion causes a mitotic arrest leading to p53-independent apoptosis. *PLK1* plays an essential role in the transition of the cell cycle from late G₂ phase, throughout mitosis to cytokinesis¹⁰ and its downregulation has been proposed to contribute to the G₂/M checkpoint.^{22–24} To determine the outcome of downregulating *PLK1* levels on cell cycle progression *PLK1* expression was silenced by siRNA. Knock-down of *PLK1* in the HCT116 (p53^{+/+}) cells (Fig. 9A) led to cell cycle arrest at both the G₁ and G₂/M phases of the cycle with a small proportion of the cells undergoing apoptosis as evidenced by flow cytometry (Fig. 9B), and PARP cleavage (Fig. 9A). The presence of increased levels of phosphorylated histone H3 in these cells, and their rounded-up appearance (Fig.

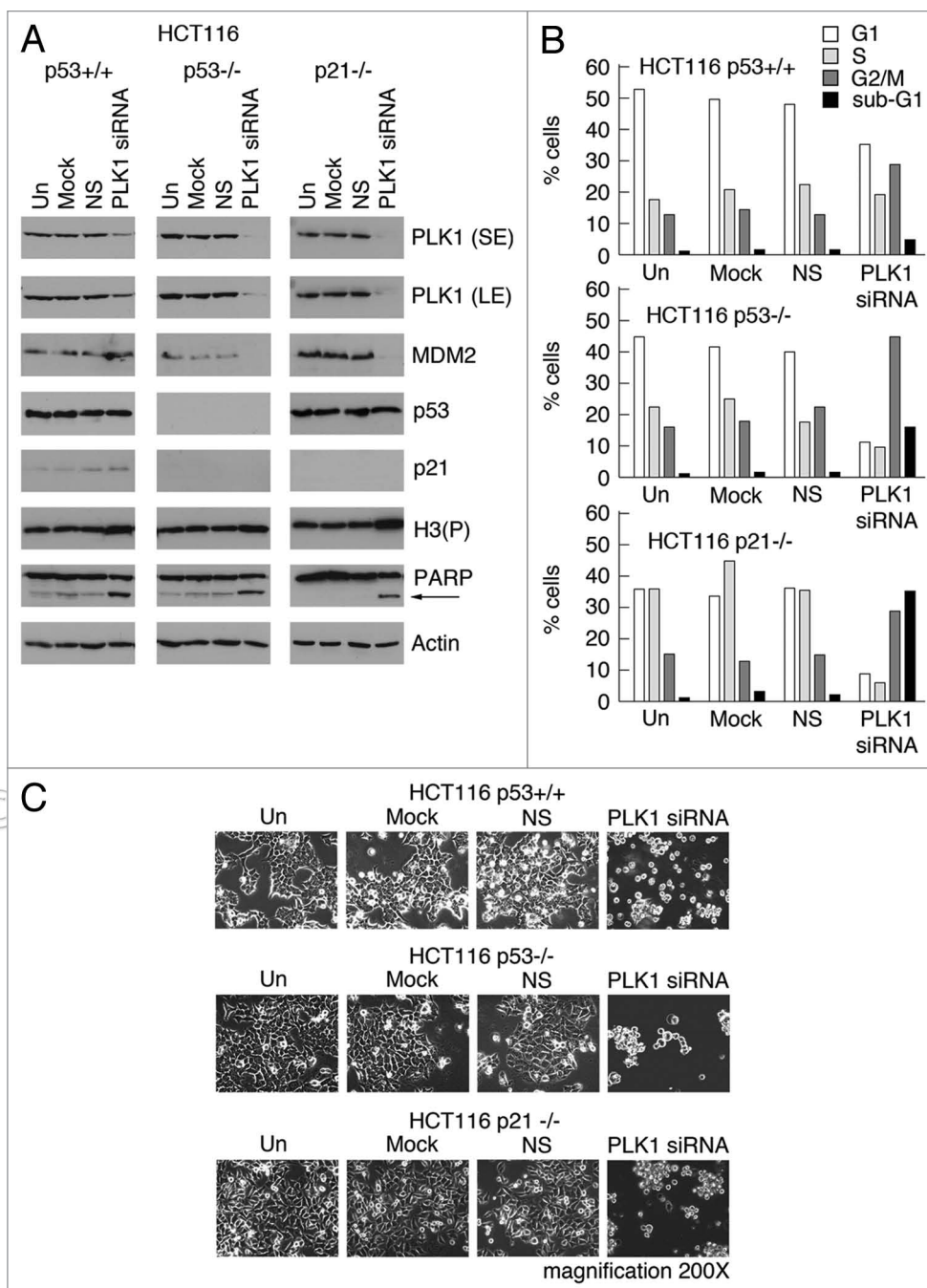


Figure 9. Silencing of *PLK1* expression leads blocks cell cycle progression and initiates apoptosis. *PLK1* expression in parental HCT116 cells, and in the p53-null and p21-null derivatives, was silenced by siRNA transfection. The cells were harvested 24 h after transfection. (A) western blotting was performed with the antibodies as indicated. (B) Cell were analysed by Flow cytometry. A set of data is shown that is representative of three independent experiments. (C) The cells were examined by phase contrast microscopy.

9C), suggested that they had entered mitosis before undergoing p53-independent apoptosis. When p53 or p21 were absent there was no G₁/S arrest with the outcome that there was a higher proportion of cells at G₂/M and a significantly increased level of apoptosis. These data suggest that p53 may play an additional role by protecting the cells from loss of *PLK1*, presumably by holding a proportion of the cells at the G₁/S boundary. The finding that

loss of PLK1 permits the cells to enter M phase but terminate in apoptosis is consistent with the idea that p53 may mediate a checkpoint that leads to cell death through the downregulation of PLK1. Alternatively, however, it is possible that a G₂/M cell cycle arrest may indeed involve p53-mediated depletion of PLK1, but may additionally require other p53-coordinated events to hold the cells at the G₂/M boundary thereby halting any commitment towards undergoing cell death.

Discussion

In the present study we show that PLK1 protein and -mRNA levels are depleted in response to DNA damage-inducing agents in a p53-dependent manner. Moreover, the use of Nutlin-3, which inhibits the p53-MDM2 interaction,³¹ or expression of p53 via an inducible system demonstrated that induction of p53 alone is sufficient to achieve this effect in the absence of DNA damage. These data suggest that PLK1 is likely to be a target of p53 not only following DNA damage but also in response to other stimuli that induce a p53 response. The data also suggest that p53 can mediate these effects by directly repressing expression of *PLK1*. In support of this idea we find that p53 is recruited to two distinct regions within the *PLK1* promoter. In one case (p53RE1) this is associated with an open chromatin structure that is unaffected by p53 stimulation. In the other case however (p53RE2) the chromatin conformation becomes closed (as evidenced by loss of histone acetylation) in response to p53 activation and increased p53 recruitment (Fig. 5). The involvement of histone deacetylase in the p53-dependent downregulation of PLK1 additionally supports a repression model (Fig. 6). At present we do not know what additional components are required for this repression mechanism, nor do we know the sequence of events involved or the means by which the repression is regulated. We have, however, been able to rule out certain mechanisms. For example, p21-dependent or -independent repression via the CDE/CHR element, while relevant to some promoters (reviewed in ref. 20), and which has previously been suggested by others to mediate the ability of p53 to regulate PLK1 levels,²⁸ does not appear to be a major contributor to repression of *PLK1* (Figs. 4, 5 and 8 and Suppl. Fig. 2). The finding that p53 could not be detected close to the transcriptional start site (Fig. 5) also suggests that the inhibition of DNA-bound NF-Y that occurs in promoters such as the CDC2 and Cyclin B2 promoters is unlikely to mediate repression of PLK1.⁷

The finding that mutation of the p53 responsive element, p53RE2, leads to a loss in basal *PLK1* expression suggests that a transcription factor that is crucial for maintaining basal PLK1 levels binds to a site that overlaps with p53RE2. It also raises the possibility that part of the repression mechanism may involve “competitive displacement” by p53, a mechanism that is known to mediate repression of several other p53-responsive promoters,^{20,36,37} in some cases (e.g., CDC25C²⁰) as part of a dual repression mechanism. Initial analysis of the p53RE2 sequence suggests a list of at least 20 different transcription factors that could bind to this region. Identifying this factor will therefore be an important goal in exploring the elements of this repression mechanism in depth.

Our data also demonstrate that PLK1 satisfies the five conditions that normally constitute a p53-responsive gene.⁵ These are: (a) presence of a demonstrable p53-responsive element in the promoter (Figs. 7 and 8); (b) downregulation at the RNA and protein level by wild type, but not mutant, p53 (Figs. 1–3 and 8); (c) p53 regulation conferred on a test gene through the responsive element (Fig. 8); (d) the presence of p53 on the endogenous promoter within the vicinity of the response element as revealed by ChIP (Fig. 5); and (e) sequence-selective binding of p53 to the RE in vitro (Fig. 7). Our data support and significantly extend previous observations suggesting that *PLK1* may be downregulated by a p53-dependent repression mechanism.^{21–24,26,27}

Several laboratories have examined the outcome of silencing expression of PLK1 and broadly agree that depletion of PLK1 leads to several defects associated with the transition through mitosis and cytokinesis, resulting in mitotic catastrophe and/or apoptosis.^{14,38–44} Interestingly, PLK1 depletion has also been reported to lead to DNA damage.^{14,41} Since p53 mediates the repression of PLK1 (both at the levels of expression and post-translational modification [this study; reviewed in ref. 22–27]), we re-examined the effects of depleting PLK1 to determine the likely consequence of its downregulation following p53 induction. Consistent with published studies, we find that PLK1 knock-down arrests the cells not in G₂ but in mitosis, as judged by flow cytometry profile, cellular morphology and the presence of phosphorylated histone-H3 (a marker for mitotic cells; Fig. 9). Notably, these cells fail to complete mitosis (as evidenced by the reduction in the G₁ peak [in the p53-null and p21-null derivatives])—i.e., they are not re-entering cycle) and undergo apoptosis, as shown by the presence of a sub-G₁ population of cells together with the appearance of cleaved PARP. These observations raise some curious questions about the impact of inducing p53 on late cell cycle events. On the one hand, the downregulation of PLK1 by p53 could lead *indirectly* to apoptosis by allowing cells to progress towards mitosis when they lack this critical mitosis orchestrator. Arguing against this, however, is the observation that downregulation of PLK1 does not lead to any significant increase in apoptosis when it is achieved through inducing p53 (as judged by the lack of any increase in the sub-G₁ population—see Suppl. Fig. 2). A more plausible explanation is that p53 stably maintains arrest in G₂ through a series of events including downregulation of CDC25C, Cyclin B1, CDC2, Stathmin, MAP4, etc., (reviewed in ref. 6 and 45) that are by necessity coordinated and inter-dependent, and that it is within this context that the inhibition of PLK1 functions as an important and integral element in this orchestration. We also note that arrest in mitosis followed by apoptosis occurs not only in the HCT116 parental cells but also in the p53-null and p21-null derivatives (Suppl. Fig. 2). Strikingly, however, the level of apoptosis is significantly greater in the absence of p53, and even greater still when p21 is absent. A protective effect of p53, consistent with these observations, has also been noted by others.^{14,27,38,41,42} We suggest, from the flow cytometry profiles in **Supplementary Figure 2B**, that the ability of p53 and/or p21 to arrest the cells in G₁ may afford this protection, at least in part, by preventing the cells from progressing to the G₂/M stages of the cell cycle where the absence of PLK1

becomes a critical problem. Additionally, however, the recent demonstration that PLK1 is required for pre-replication complex formation during DNA synthesis, and consequently that depletion of PLK1 leads to DNA damage,^{14,41} suggests that p53 may be alerted to abnormally low PLK1 levels through the DNA damage pathways and provides additional protection by blocking entry into and/or progression through S phase.

Our data are also consistent with the growing awareness that p53 status may have a profound influence on PLK1 levels in tumors.²⁷ Our findings thus provide mechanistic insight into how mutation of p53 or loss of p53 function, during tumorigenesis can lead to, or contribute towards, unregulated PLK1 levels and, consequently, to an augmented hyper-proliferative drive. They also support the idea, expressed by other researchers,^{27,38,42} that an intact p53 pathway may assist the selective treatment of tumor cells with PLK1 inhibitors by affording a level of protection to normal cells.

Materials and Methods

Cell culture. Cells were maintained in McCoy's 5a medium (Biosera; HCT116 [p53^{+/+}, p53^{-/-} and p21^{-/-}] colon carcinoma cells³⁰) were maintained in McCoy's 5a medium (Biosera) or Dulbecco's modified Eagle's medium (Biosera; OSA, U2OS, MCF-7 and SAOS-2), supplemented with 10% heat-inactivated fetal bovine serum (Biosera), penicillin (100 IU/ml)/streptomycin (100 µg/ml) (Invitrogen) and 2 mM Glutamine (Invitrogen). Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Flow cytometry. Cells were treated with 5 µM Nutlin-3 or untreated as control, for 24 h. Cells were analyzed by flow cytometry following labeling with BrdU and propidium iodide as described previously.³²

Luciferase reporter assays. A *PLK1*-luciferase reporter plasmid was constructed by cloning 2.3 kb of the human *PLK1* promoter (base numbers 1,590–3,893 from the Genbank sequence X90725) upstream of the firefly *luciferase* gene in the plasmid pGL3-basic (Promega). Cells (3 x 10⁴/well) were seeded onto a 24-well plate and transfected in triplicate using Fugene (Roche) as indicated in the figure legends. Transfected cells were harvested 24 hr post-transfection by adding 100 µl of passive lysis buffer (Promega) per well. 20 µl of protein extract was analyzed in a luminometer using the Dual Luciferase Reporter Assay Kit (Promega). Variations in transfection efficiencies were corrected by determining the *Renilla luciferase* activity of the sample.

Site-directed mutagenesis. Point mutations at the CDE/CHR site in the *PLK1* promoter-luciferase plasmid were generated using the Stratagene Quik-Change system according to the manufacturer's instructions. The complementary mutant oligonucleotides were as follows with the mutant bases shown underlined:

Coding strand oligonucleotide: 5'-GTA ACG TTC CCA TAT CCG CGT TCA CAT TCG GGG AGG AG-3'

Non-coding strand oligonucleotide: 5'-CTC CTC CCC GAA TGT GAA CGC GGA TAT GGG AAC GTT AC-3'.

Gene silencing using siRNA. HCT116 and U2OS cells were transfected with PLK1 or p21 siRNA (ON-TARGETplus SMARTpool L-003290-00 or L-003471-00 respectively Dharmacon) using Lipofectamine (for U2OS cells) or by reverse transfection using Lipofectamine RNAiMAX Transfection Reagent (for HCT116 cells) in a 6-well plate according to the manufacturers' instructions.

Real-time (Taqman) RT-PCR. Total RNA was isolated from cells using the RNeasy Mini Protocol according to the manufacturer's instructions (Qiagen Ltd.). cDNA was synthesized using standard reverse transcription PCR techniques. Steady-state levels of mRNA encoding PLK1 and MDM2, were determined by real-time quantitative PCR as described previously.⁴⁶ The primers and probe for PLK1 amplification were as follows.

PLK1 Forward primer: 5'-GGA TCA CAC CAA GCT CAT CTT G-3'

PLK1 Reverse primer: 5'-CCC GCT TCT CGT CGA TGT-3'

PLK1 Probe: 5'-CCC ACT GAT GGC AGC CGT GAC C-3'

The primers and probes for MDM2 were as follows.

MDM2 Forward primer: 5'-CAA GTT ACT GTG TAT CAG GCA GGG-3'

MDM2 Reverse primer: 5'-CCT TCC ATC ACA TTG CAA CAG A-3'

MDM2 Probe: 5'-TTG GAA ATG CAC TTC ATG CAA TGA AAT GAA TCC-3'

Antibodies and western blot analysis. SDS-PAGE and western blotting was carried out using standard conditions. Nitrocellulose membranes were probed for the presence of PLK1 (antibodies PL2 and PL6, Zymed Laboratories Inc.), p53 (antibody DO1, Moravian Biotechnology; or CM-1,⁴⁷), p53 phospho-Ser15 (Ab-3, Calbiochem), MDM2 (SMP14, D12, Santacruz Biotechnology, Inc.; or 4B2, Moravian Biotechnology), p21 (H-164, Santacruz Biotechnology, Inc.), 14-3-3σ (sc-7683, Santacruz Biotechnology, Inc.), phospho-Histone H3 (06-570, Upstate Biotechnology), acetyl-lysine (4G12, Upstate Biotechnology), HDAC1 (sc-6298, Santacruz Biotechnology, Inc.) or actin (20-33, Sigma-Aldrich). Secondary antibodies used were HRP-conjugated rabbit anti-mouse (DAKO), -goat anti-rabbit (DAKO) or -donkey anti-goat (sc-2020, Santacruz Biotechnology, Inc.). Proteins were detected by enhanced chemiluminescence according to the manufacturer's instructions (Pierce Biotechnology, Inc.).

Cell lysis. Cells were lysed in 50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.25% Sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, 50 mM Sodium fluoride, 1 mM Sodium orthovanadate, 1 mM Benzamidine and a protease inhibitor cocktail (Calbiochem), left on ice for 10 mins, followed by centrifugation at 13,000 rpm for 10 mins at 4 degrees. The soluble fractions were collected.

Infection with recombinant adenovirus. Recombinant viruses Ad-MDM2, Ad-GFP and Ad-CON have been described elsewhere.⁴⁸ HCT116^{-/-} cells were grown to 80% confluency and infected at an MOI of 50, followed by an incubation period at 37°C for 4 h (35). Cells were then treated with 10 µM Nutlin-3,³¹ and incubated at 37°C for a further 24 h. Extracts were analyzed by western blotting.

Quantitative chromatin immunoprecipitation (ChIP). The promoter sequence of human *PLK1* (accession number X90725) was screened for potential p53 binding sites using in-house software. This approach identified potential p53 binding sites at positions, relative to the transcriptional start site, -2,086 to -2,016 (p53 binding site-1 [p53RE-1]) and -816 to -785 (p53 binding site-2 [p53RE2]). The sequence of these regions is given in **Figure 5A**. Primers and probes for quantitative chromatin immunoprecipitation analysis were designed using Primer Express software (Applied Biosystems) to allow amplification of the regions encompassing the two potential p53 binding sites and, additionally, the CAAT and CDE/CHR signals (both of which are located close to the transcriptional start site). The primers and probes were as follows:

p53 binding site-1 Forward primer: 5'-CAC TGT GCC TAA ACT GTT CGT ACA-3'

p53 binding site-1 Reverse primer: 5'-CAC ATA TGA AAA TCT GAC TCC CAG AA-3'

p53 binding site-1 Probe: 5'-TGT GGG TTA TGC CCA ACA TCT GCT TTC-3'

p53 binding site-2 Forward primer: 5'-TGC TAG GCA TTC TTC CAA CCT T-3'

p53 binding site-2 Reverse primer: 5'-GAA TTT CCC AGG GCC TAA AAT AG-3'

p53 binding site-2 Probe: 5'-CCT CCC TCT GAC CAA GAA ACT GAG TGT CC-3'

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CAATT box Forward primer: 5'-ATC CCC GCG GCC AAT-3'

CAATT box Reverse primer: 5'-TCC TCC CCG AAT TCA AAC G-3'

CAATT box Probe: 5'-CGC GCA GGC TTT TGT AAC GTT CCC-3'

p21 promoter, distal p53 binding site Forward primer: 5'-GTG GCT CTG ATT GGC TTT CTG-3'

p21 promoter, distal p53 binding site Reverse primer: 5'-CTG AAA ACA GGC AGC CCA AG-3'

p21 promoter, distal p53 binding site, probe: 5'-TGG CAT AGA AGA GGC TGG TGG CTA TTT TG-3'

For the quantitative ChIP analysis, U2OS or HCT116 cells were treated with up to 50 μ M Etoposide, 20 μ M Cis-platin or 10 μ M Nutlin-3 for 6 or 8 h and chromatin immunoprecipitation was performed as described previously.^{32,49}

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/McKenzieCC9-20-Sup.pdf

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